METAL BINDING PROPERTIES OF P97 AND THE TRAFFICKING OF A NOVEL IRON INTERNALIZATION PATHWAY BY GPI-ANCHORED P97

by

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Abstract

Iron is an essential for cellular metabolism yet excessive iron can lead to deleterious oxidative damages in the cells. Since humans have no physiological pathways for iron excretion, dietary iron absorption in the intestine is tightly regulated. Although the essential nature of iron for normal cellular functions is beyond doubt, only in the last two decades has its uptake and regulation been relatively well understood.

The transferrin/ transferrin receptor-independent iron transport pathways play a major role in iron overload diseases such as hemochromatosis. However, very little is known about the molecular details of these pathways. P97, also known as melanotransferrin, is an example of iron binding proteins that mediates such pathways. P97 is a member of the transferrin family, which includes serum transferrin, lactoferrin and ovotransferrin. Unlike members of the transferrin family, which are all serum proteins, p97, in addition to being a serum protein, also exists as a glycosylphosphatidylinositol-anchored protein. The primary iron-binding site in the Nterminal lobe is highly conserved among the other transferrin members. However, the iron binding site at the C-terminal lobe of p97 is not. This leads to the question as to whether p97 is a functional iron transport protein. In addition, the iron binding affinity of p97 has never been ascertained. Therefore, an aspect of this thesis was to determine the binding affinity of iron to p97 and to establish whether the C-lobe of p97 can bind iron. The results in this thesis will demonstrate that the iron binding affinity for the N-lobe of p97 is 2.2×10^{17} M⁻¹ at 25 °C. In addition, the data will show that C-lobe is able to bind iron albeit very weakly.

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Metals such as iron, copper, aluminum and zinc have been implicated in the pathology of several neurological disorders. The expression of p97 in the brain capillary endothelium and the elevated level of serum p97 associated with Alzheimer s disease have led to the hypothesis that p97 may play an important role in disease progression of the disease. Therefore, another goal of this study was to establish whether p97 can bind metals such as copper, aluminum and zinc. The results will show that copper, aluminum and zinc are able to interfere with iron binding to GPI-anchored p97 and soluble p97 is able to bind to these metals as shown by urea polyacrylamide gel electrophoresis.

Though the internalization pathways of many GPI-anchored proteins have been studied, debate still exists as to whether caveolae or clathrin-coated vesicles are involved in their uptake. Surprisingly, very little is known about the fate of the ligands internalized by GPI-anchored molecules. Also, there is controversy as to whether iron bound to p97 can be donated to ferritin. Therefore, another aspect of this thesis was to examine the uptake of GPI-anchored p97 and its ligand, iron. The results from confocal immunofluorescence microscopy and sub-cellular fractionation demonstrate that GPI-anchored p97 bound with iron is endocytosed into SK-MEL 28 cells via caveolae and traffics to early endosomes. In addition, the data also shows that GPI-anchored p97 is present in the same vesicles as Nramp2, a metal transporter thought to mediate iron transport across the vesicular membrane into the cytoplasm. Furthermore, it is shown that iron taken up by p97 becomes bound by the iron storage molecule, ferritin. The data establishes that iron bound to GPI-anchored p97 is internalized through caveolae and donated to ferritin for cellular metabolism and supports an improved understanding of

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both GPI anchor mediated uptake of ligand and the novel transferrin/ transferrin receptorindependent mechanism of iron uptake.

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List of Abbreviations:

Al	Aluminum
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
СНО	Chinese hamster ovary
Cu	Copper
Cy5	Cyanine 5
Dcytb	Duodenal ferric reductase
DMEM	Dulbecco modified eagle s media
DMSO	Di-methyl-sulfoxide
DMT-1	Divalent metal transporter
DNA	Deoxyribonucleotide
DSC	Differential scanning calorimetry
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EEA1	Early endosome associated antigen 1
EGFP	Enhanced green fluorescence protein
Fe	Iron
GPI	Glycosylphosphatidylinositol
HEPES	2-Hydroxyethyl-piperazine-2-ethanesulfonic acid
HFE	Protein responsible for hereditary hemochromatosis
HLA	Human Leukocyte Antigen
hpx	Hypotransferrinemic

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H_2O_2	Hydrogen	peroxide
		-

- HO• Hydroxyl radical
- HO⁻ Hydroxyl ion
- IMDM Iscove's modified dulbecco media
- IREG1 Iron regulated transporter
- MEM Minimum essential media
- NEAA Non essential amino acids
- mRNA messenger ribonucleotide
- Nramp2 Natural resistance associated macrophage protein 2
- NRS Normal rabbit serum
- NTA Nitrilotriacetate
- $O_2 \bullet^-$ Superoxide
- O₂ Oxygen
- P97⁺TRVb Human p97 transfected TRVb
- PBS Phosphate buffered saline
- PEG Polyethylene glycol
- PI-PLC Phosphatidyl-phosphoinositol lipase C
- TfR Transferrin receptor
- TfR⁺TRVb Human transferrin receptor transfected TRVb
- TRVb Chinese hamster ovary cell with defective endogenous transferrin receptor
- UV Ultraviolet
- XANES X-ray absorption near edge structure
- Zn Zinc

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Dedication

This thesis is dedicated to my mother, Jenny S.J. Lam and my father Stanley Y.S. Tiong. Your loves and support have made this journey possible and for this I am eternally grateful.

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Chapter 1 Introduction

I. A brief history of iron in medicine

Mesopotamian civilization long ago regarded iron as the "metal of heaven". The belief in the celestial origin of the ore, which has its inference with the stars, is instilled in its Greek name--*sidero*. The shower of meteorites, which indeed contained iron, made the Greeks and the Romans believe that iron was a gift from the heavens.

Ancient physicians who observed the similarity in the color of blood and iron rust established the association of iron with life. *Ebers Papyrus*, the oldest Egyptian pharmacopoeia manuscript dated 1500 BC, recorded the use of iron as a remedy for baldness (Ebbell, 1937). Hippocrates, the great Greek physician, believed in using iron salts for relieving gastrointestinal tract ailments. When Arabic civilizations blossomed after the fall of the Roman Empire, the famous Arabic physician Ibn Sina compiled a massive catalogue of 10th century medical knowledge called the Al-Qanun, also known as the Canons of Medicine in the West. He cited the use of iron for improving incontinence of urination, restraining menstrual flow and restoring libidinous potency and strength to men. In the 17th century, a mysterious illness termed chlorosis manifested in menstruating girls and young women. A French physician named Pierre Blaud recognized the iron deficiency in these chlorotic girls and prescribed oral iron salts as treatment for the disease. Although iron was not universally given to treat chlorosis, the disease disappeared before World War II, presumably due to improved diet.

Iron deficiency continues to be an important public health concern even today despite the fortification of food supplies and the increased use of iron supplements (Baynes and Bothwell, 1990). Iron deficiency can lead to impaired immune functions,

poor cognitive development, and other pathologies (Beard et al., 1996; Dallman, 1986). Paradoxically, too much iron is harmful due to its role in the generation of free radicals, which can lead to oxidative injuries such as lipid peroxidation and DNA damage (McCord, 1998). Among the different iron overload disorders, hereditary hemochromatosis is the most studied disorder. Hereditary hemochromatosis was first recorded by French physician Armand Trousseau in 1865 (Trousseau, 1865) and the term hemochromatosis, used to describe the disease of iron overload, was coined by von Recklinghausen in 1889 (Recklinghausen, 1889). A breakthrough came in 1935 when an English physician recognized that the inborn error in iron metabolism is inherited through a single gene defect (Sheldon, 1935). The cloning of the gene responsible for the disorder, hfe, and the identification that mutations in the HFE protein responsible for this disorder, have lead to a breakthrough in the understanding of the pathology of the disease (Feder et al., 1996).

Advances in techniques and medical research have led to an increased understanding of the role of iron in health and disease. Recently, various genes involved in iron uptake and iron metabolism were identified. Exciting discoveries are continually being made as novel proteins are identified and researchers are piecing them into the puzzle of iron metabolism.

II. Roles of iron in cellular metabolism

Iron is an essential element for cellular metabolism. Except for some species of *Lactobacilli*, all organisms require iron for existence and proliferation (Archibald, 1983). However, if the amount of iron exceeds the requirement of the cell, it can be deposited

into vital organs and tissues. Excess iron deposition is dangerous due to its role as a catalyst in generating free radicals. Haber and Weiss were the first to recognize that the highly reactive hydroxyl radical (HO•) could be generated from the interaction between superoxide (O_2 •) and hydrogen peroxide (H_2O_2) (Equation 1, also known as Haber-Weiss reaction). They also realized that this reaction is thermodynamically unfavorable in a biological system unless a metal ion catalyst is present (Haber and Weiss, 1934). The Haber-Weiss reaction can be broken down into two chemical reactions with iron acting as the catalyst (Equations 1.1 and 1.2). The net reaction (Equation 1) is considered to be the major mechanism by which highly reactive HO• is generated in a biological system (Liochev, 1999).

$$O_2 \bullet^- + H_2 O_2 \to O_2 + HO^- + HO \bullet$$
 (1)

$$Fe^{3^+} + O_2 \bullet^- \to Fe^{2^+} + O_2 \tag{1.1}$$

$$Fe^{2^+} + H_2O_2 \rightarrow Fe^{3^+} + HO^- + HO^-$$
(1.2)

HO• is one of the reactive oxygen species that can potentially cause serious damage in the cell. For example, guanine bases of DNA are particularly sensitive to oxidation and if this damage is not repaired, it can impair DNA function (Lindahl and Wood, 1999). The double bonds in polyunsaturated fatty acids are also susceptible to free radical attack. The resultant lipid peroxyl radical can attack another fatty acid thereby setting up a chain reaction. As a result, the normal functions of enzymes or proteins that reside in the plasma membrane are affected. Proteins too are susceptible to oxidative damage. Although most oxidized or damaged proteins are promptly removed by proteolysis (Grune et al., 1997), some can slowly accumulate with time and thereby contribute to plaque deposition as seen in various diseases such as arteriosclerosis and

neurodegenerative diseases (Stohs, 1995; Stohs and Bagchi, 1995). Therefore, although iron is an essential element in cellular metabolism, the *ying* side of the metal has to be controlled so that the *yang* side can be fully exploited for cellular functions.

The role iron plays in cellular metabolism stems from its capacity to accept and donate electrons readily by inter-converting between the ferric and the ferrous forms (Beinert, 1976). This property makes iron a cofactor in molecules such as cytochromes, oxygen-binding proteins such as hemoglobin and myoglobin and other essential enzymes. Table I is a list of some of the iron-containing enzymes or proteins involved in mammalian cellular functions such as energy generation in the mitochondrial electron transport chain, detoxification of free radical, cellular proliferation and regulation of gene expression.

The first class of iron-containing proteins, which are involved in electron transfer, usually contains one or more iron-prosthetic groups such as heme, flavin, and iron-sulfur clusters. One function of proteins containing heme and iron sulfur clusters is electron transport in the respiratory chain of the mitochondria. They can provide orbital overlap pathways for the transfer of electronic charges (Marcus et al., 1980). Some of the mitochondrial iron proteins such as NADH-ubiquinone reductase, ubiquinol: cytochrome c reductase and cytochrome c oxidase also participate in the transfer of protons across the mitochondrial matrix (Rich, 1984). The electrochemical gradient generated by the transfer of protons can then be used for the generation of ATP (Reid et al., 1966). The second class of iron containing proteins interacts directly with oxygen. These proteins, which include dioxygenases, monooxygenases, peroxidases and catalases, are usually involved in cellular detoxification. The dioxygenases catalyze reactions that incorporate

Electron transport and cellular respiration: Cytochrome C Cytochrome oxidase Ubiquinol: cytochrome c reductase NADH: ubiquinone reductase Succinate: ubiquinone reductase Cellular detoxification and oxygen-dependent reactions: Dioxygenases Monooxygenases Peroxidases Catalase Ribonucleotide reductase Cellular proliferations: Iron regulatory protein 1 Post translation regulation of gene expression: Iron regulatory protein 2 Other iron containing proteins: Redox enzymes (e.g. Xanthine oxidase) Non-redox enzymes (e.g. Aconitase) Iron transport (e.g. Transferrin, lactoferrin and p97) Iron storage (e.g. Ferritin) Reversible oxygen binding protein (e.g. Hemoglobin and myoglobin) .

Table I: Examples of some iron proteins involved different mammalian cellular functions

both atoms of molecular oxygen into the substrate (Lange and Que, 1998) while the monooxygenases are required for processes where molecular oxygen is used in hydroxylation reactions, often to break an aromatic ring (Karuzina and Archakov, 1994). The peroxidases and catalases oxidize toxic compounds at the expense of reducing H_2O_2 to H_2O . This reaction provides protection to the cell from the toxic effects of hydrogen peroxide. The third class of iron-containing enzymes includes ribonucleotide reductase, which is essential for synthesis of DNA during cellular proliferation (Sahlin and Sjoberg, 2000; Stubbe, 2000). The fourth class of iron containing proteins, which are involved in regulating gene expression, includes the iron regulatory protein 1 and iron regulatory protein 2. They regulate expression of genes that contain the specific iron regulatory element in their messenger RNA (mRNA) (Eisenstein, 2000). Finally, there is a large category of iron containing proteins, which do not have any functional role in redox reactions. For example, hemoglobin and myoglobin use the heme prosthetic group mainly to bind oxygen reversibly and deliver oxygen to specific sites, and iron transport proteins such as ferritin, transferrin, lactoferrin and p97.

III. Diseases related to iron metabolism

Due to the toxicity of free iron, the level of iron in an organism is tightly regulated through the gene expression of proteins involved in iron metabolism. Any aberration or disruption to the intricate balance contrived by these proteins would inevitably results in clinical manifestations that usually carry severe consequences. Micro-molar levels of non-transferrin bound iron are detected in organs of patients suffering from iron overload disorder (Batey et al., 1980; Grootveld et al., 1989;

Gutteridge et al., 1985). For example, in some iron overload disorders such as hereditary hemochromatosis and African dietary iron overload, there is excess iron deposition in vital organs such as liver, pancreas and heart. The increased iron load in these tissues ultimately leads to organ failure if the disease is not treated. The transferrin/ transferrin/ receptor is thought to be the major pathway for cellular iron uptake. The details regarding the iron uptake pathway mediated by transferrin/ transferrin receptor will be discussed in details later in the chapter. However, it is unclear whether transferrin/ transferrin receptor is responsible for the increased deposition of non-transferrin bound iron in the organs of hereditary hemochromatosis patients. Recently, the existence of transferrin/ transferrin receptor independent iron uptake pathways has been described (Conrad and Umbreit, 1993; Conrad et al., 1994; Craven et al., 1987; Egyed, 1988; Kennard et al., 1995; Morgan, 1988; Richardson and Baker, 1991a; Yu et al., 1998). There is increasing evidence to support that these pathways might be responsible for the iron burden in hereditary hemochromatosis patients (McNamara et al., 1999). Table II is a list of diseases related to iron metabolism with an emphasis on diseases relating to iron overload. Each of the diseases will be discussed briefly.

A. <u>Hereditary hemochromatosis</u>

Hereditary hemochromatosis is an autosomal recessive disorder with high prevalence in Causcasians. An estimated 10% of the population is heterozygous for the gene, with 1 in 400 having the disease (Edwards et al., 1988). It is characterized by excessive iron deposition in various vital organs that leads to severe multi-organ failure including diverse pathologies in the liver, pancreas and heart. Liver damage, including

Hereditary Hemochromatosis	Iron overload in patients carrying specific mutations in the HFE gene
Non-HFE hemochromatosis	
Juvenile hemochromatosis	Iron overload with early onset by the age of 30 with an accelerated rate, the HFE gene is normal
Neonatal hemochromatosis	Lethal iron overload resulted in liver failure in neonates
African Iron overload	Iron overload related to genetic predisposition in 10% of the sub-Saharan African population and is exacerbated by dietary iron intake
Transferrin receptor 2 related	Non HFE iron overload linked to a Y250X amino
iron overload	acid substitution in the transferrin receptor 2 protein
Aceruloplasminemia	Iron accumulation due to aberration in the ceruloplamin protein, iron accumulates in the reticuloendothelial cells and hepatocytes. Cellular iron uptake is normal
•	but iron egress is impaired
Neurodegenerative disease	

Table II: Diseases related to mammalian iron metabolism

Alzheimer s disease

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Metals such as iron, copper, aluminum and zinc have been implicated in the disease progression

Parkinson s disease	Oxidative stress caused by iron has been linked to
	neuronal death
Hallervorden Spatz Syndrome	Caused by a defect in pantothenate kinase 2 gene
	which result in increased free radical stress in regions
	of the brain with high iron accumulation
Neuroferrinopathy	Insertion of adenine in the carboxy terminal of the L
	chain of ferritin partially compromises the structure
	and function of ferritin
Hyperferritinemia	Point mutation of in the IRE of the L chain ferritin
	mRNA which leads to increased serum ferritin level
	with bilateral nuclear cataracts
Atransferrinemia	Disruption of transferrin gene, which ultimately lead
	to iron deposition in various
	tissues.
Diseases related to mitochondria	

iron metabolism

•

Friedreich's ataxiaIron deposition in the neuronal cells and cardiac
myocytes due to disruption of iron homeostasis in
mitochondriaX-linked sideroblastic anemiaIron deposition in mitochondria of erythrocyte

precursors and neuronal cells as a result of missense mutations in the eALAs gene at Xp11.21, which affects the

biosynthesis of heme

•

hepatocellular carcinoma, is the major cause of mortality. The gene that is responsible for causing hereditary hemochromatosis is a human leukocyte antigen (HLA) related protein called HFE. Like other HLA class-I related proteins, HFE associates with β 2microglobulin on the cell surface (Feder et al., 1996). However, instead of its classical function in peptide binding in its α 1 and α 2 domains, crystal structure shows that HFE forms a high affinity complex with transferrin receptor (Lebron et al., 1998).

There are several amino acid substitutions in the HFE protein that have been shown to associate with the pathology of hereditary hemochromatosis. The most common one is the C282Y amino acid substitution in the α 3 domain. Patients who are homozygous for this mutation usually develop iron overload. A subset of heterozygotes is also affected with varying degree of severity (Edwards et al., 1998; Olynyk et al., 1999). In vivo experiments in the HFE knockout mice or mice carrying the C282Y HFE transgene, show that these mice exhibit severe iron overload. The change of the cysteine residue at position 282 prevents the formation of the disulfide bond in the α 3 domain of HFE. This prevents the protein from folding properly and associating with β 2microglobulin, which ultimately eliminates the expression of HFE on the cell surface (Feder et al., 1997; Waheed et al., 1997). There are several hypotheses on how HFE affects iron metabolism. HFE is shown to interact directly with transferrin receptor (Feder et al., 1997; Parkkila et al., 1997). Physical association of HFE with transferrin receptor is required for trafficking of transferrin receptor to the cell surface (Gross et al., 1998; Salter-Cid et al., 2000a). However, it is thought that the interaction between HFE and transferrin receptor decreases the binding affinity of transferrin to transferrin receptor (from Kd of 5 nM in the presence of HFE to Kd of 12-75 nM in the presence of HFE)

(Feder et al., 1997). Nonetheless, since serum transferrin is present in the micro-molar range, the binding of transferrin to transferrin receptor is saturated even in the presence of HFE (Salter-Cid et al., 2000b). Others have also shown that direct interaction of HFE with transferrin receptor reduces the internalization rate of transferrin/transferrin receptor complex (Salter-Cid et al., 1999; Salter-Cid et al., 2000a). Therefore, when HFE fails to complex with transferrin receptor, such as in the case of the C282Y HFE, the increased rate of transferrin/transferrin receptor uptake can explain the excess iron accumulation in tissues. Although there is a wealth of evidence that the C282Y mutation indeed causes hereditary hemochromatosis, more experiments are needed to define how HFE regulates iron absorption. Other polymorphisms such as the S65C, I105T and G93R and H63D in the HFE protein have been shown to associate with varying degree of iron overload (Barton et al., 1999; Mura et al., 1999). However, the molecular details of how these different HFEs result in iron overload are still under investigations.

Recently, a new homologue of transferrin receptor, transferrin receptor 2, was identified. The human transferrin receptor 2 shares 66% similarity and 46% identity in protein sequence on the extracellular domain with the human transferrin receptor 1 (In the context of transferrin receptor 2, the first identified human transferrin receptor will be notified as human transferrin receptor 1) (Kawabata et al., 1999). Transferrin receptor 2 can bind to transferrin at a lower affinity and internalize it (West et al., 2000). The transferrin receptor 2 also showed a preferential expression in the liver where the expression of transferrin receptor 1 is lower (Fleming et al., 2000). Interestingly, the transferrin receptor 2 gene has been implicated in another form of hemochromatosis, which is not related to the classical hereditary hemochromatosis. A homozygous

nonsense mutation, Y250X, which resulted in a pre-matured stop codon in the transferrin receptor 2 gene was shown in several families affected with non-classical hereditary hemochromatosis (Barton et al., 2001; Camaschella et al., 2000). Nevertheless, the exact mechanism causing the iron overload has not been characterized.

B. Juvenile hemochromatosis

Juvenile hemochromatosis is an autosomal recessive iron overload disorder that is clinically and genetically distinct from hereditary hemochromatosis. Juvenile hemochromatosis has a much earlier onset before the third decade of the life of patients affected. The disease affects both male and female equally with the same tissue distribution as classical hereditary hemochromatosis, but iron deposition in juvenile hemochromatosis occurs at greatly accelerated rate (Camaschella et al., 1997). These patients also present a more severe case of endocrine dysfunction, joint disease and cardiac abnormalities. The locus for juvenile hemochromatosis has been located at the long arm of chromosome 1q (Camaschella et al., 1999; Roetto et al., 1999).

C. <u>Neonatal hemochromatosis</u>

Neonatal hemochromatosis is characterized by abnormal hepatic iron overload in newborn infants. In neonates, if left untreated, the disease often results in an acute, and frequently lethal liver failure. Neonatal hemochromatosis is not a homogenous disorder but rather a collection of disorders because it is often associated with other congenital anomalies (Johal et al., 1998; Vohra et al., 2000). The gene responsible for this disorder has yet to be identified.

D. African dietary iron overload

African dietary iron overload is associated with increased iron intake from a fermented maize beverage. The disorder occurs mainly in sub-Saharan African population with a genetic predisposition involving a non-HLA-linked gene, discovered through pedigree analysis of affected African families (Kasvosve et al., 2000; McNamara et al., 1999). The pattern of iron overload is different from the classical hereditary hemochromatosis. Hepatic Kupffer cells and hepatocytes contain substantial amount of iron. As a result, liver disease is the predominant organ manifestation; cardiomyopathy and diabetes are seen less often (Gangaidzo et al., 1999).

E. <u>Neurodegenerative diseases</u>

a. <u>Alzheimer s disease</u>

Alzheimer s disease is a neurodegenerative disease characterized by the deposition of β -amyloid plaques in the brain. β -Amyloid deposits in Alzheimer s disease have been shown to contain advanced glycosylation end products, copper and iron ions (Atwood et al., 2000; Lovell et al., 1998; Sasaki et al., 2001; Yan et al., 1996). Recent data have suggested that metals such as copper, zinc and iron can precipitate the formation of β -amyloid plaques (Bush, 2000; Bush et al., 1994; Loske et al., 2000). There are also evidences to support that the over expression of heme degradation enzyme, heme-oxygenase-1 (HO-1) contributes to the pathological iron deposition in Alzheimer s disease (Schipper, 2000; Schipper et al., 2000). In addition, the serum concentrations of one of the transferrin family members, p97, is elevated in Alzheimer s disease patients

compared to the age matched control group (Kennard et al., 1996; Kim et al., 2001). The correlation between iron metabolism and Alzheimer s disease is further supported by the fact that the C2 allele of the transferrin gene is more predominant in Alzheimer s disease patients than the control group. The presence of the C2 allele of the transferrin gene has been implicated in the early onset of Alzheimer s disease (van Rensburg et al., 2000).

b. <u>Parkinson s disease</u>

Another neurodegenerative disorder associated with iron overload is Parkinson s disease. The iron content of the brain of Parkinson s disease patients is elevated (Sofic et al., 1988). There is also evidence for increased iron uptake in the dopaminergic nerves of Parkinson s disease patients (Faucheux et al., 1995). The ultimate cause of neuronal death is unclear; nevertheless, a growing body of evidence suggests that increased oxidative stress caused by iron and other factors is one of the main culprits.

c. <u>Hallervorden Spatz syndrome</u>

Hallervorden-Spatz syndrome is a rare autosomal recessive disorder clinically characterized by Parkinsonism, cognitive impairment and progressive dementia. In addition to the dementia, this disorder is also characterized by iron deposition in the globus pallidus, caudate, and substantia nigra areas of the brain (Jankovic et al., 1985; Neumann et al., 2000). However, iron overload is not observed in tissues outside the central nervous system. The symptoms typically surface in the first or second decade of life and the patient usually succumbs to the disease by age 30 (Angelini et al., 1992). Recently, the gene responsible for this disorder has been identified. Missense mutations

in the pantothenate kinase 2 result in the metabolic error in pantothenate metabolism (Zhou et al., 2001). The authors proposed that the defects in the retina and basal ganglia specific pantothenate kinase 2 enzyme gave rise to a secondary excess of metabolites such as cysteine and N-pantothenoyl-cysteine. Cysteine undergoes rapid autooxidation in the presence of iron, which results in free radical production, further stressing the cell with high iron deposition in the affected brain regions (Yoon et al., 2000). Patients with Hallervorden-Spatz Syndrome also suffered from retinopathy as a result of the mutations in pantothenate kinase 2 gene. It may be possible to therapeutically deliver phosphophopantothenate or an alternative intermediary compound to cells in the affected tissues to bypass the enzymatic defect in the pantothenate metabolism in order to alleviate the disease symptoms.

d. Neuroferrinopathy

Neuroferrinopathy was recently identified as a dominantly inherited late onset basal ganglia disease with extrapyramidal features similar to those of Huntington s disease or parkinsonism (Curtis et al., 2001). By linkage analysis, the gene responsible for the disease was identified as ferritin light chain. Brain histochemistry of the neuroferrinopathy patients showed abnormal aggregates of ferritin and iron in the basal ganglia region. An adenine insertion in the carboxy terminal of the ferritin light chain may cause a conformational change and partially compromise the structure integrity of ferritin. As a result, instead of complimenting the ferritin heavy chain in the role of iron storage, irons are released from ferritin and cause toxic free radical reactions in the diseased brain.

F. Atransferrinemia

Atransferrinemia is a rare genetic disorder in which plasma transferrin is absent or is present at very low concentrations (Huggenvik et al., 1989). The patients need to be given transferrin infusions or blood transfusions for survival. Even though these patients lack transferrin in the plasma, dietary iron can be absorbed as non-transferrin bound iron (Hamill et al., 1991). Most of the non-transferrin bound iron is deposited in vital organs such as the liver, pancreas, heart, thyroid and kidney through transferrin independent pathways (Beutler et al., 2000; Hamill et al., 1991; Huggenvik et al., 1989). However, none of this non-transferrin bound iron can be used for erythropoiesis and therefore patients suffered from severe microcytic hypochromic anemia (de Silva et al., 1996)

G. <u>Aceruloplasminemia</u>

Aceruloplasminemia is a rare genetic disorder caused by a lack of ceruloplasmin ferroxidase activity due to mutations in the ceruloplasmin gene (Gitlin, 1998; Harris et al., 1998). The ferroxidase activity of ceruloplasmin is required for the release of iron from the cells. In the absence of functional ceruloplasmin, there is marked iron accumulation in the liver, pancreas and brain with lesser overload seen in the spleen, heart, kidney and thyroid. Patients with aceruloplasminemia usually develop diabetes mellitus and progressive neurodegeneration of the retina and basal ganglion suggesting that ceruloplasmin is essential for the maintenance of iron homeostasis in the central nervous system. (Harris et al., 1995). A distinction between iron overload in aceruloplasminemia and iron overload in hereditary hemochromatosis and African dietary iron overload is the involvement of the central nervous system in patients with aceruloplasminemia (Klomp et al., 1996).

H. Hyperferritinemia

Hyperferritinemia is an autosomal dominant disorder characterized by moderately increased serum L-ferritin concentration. This disorder is a result of multiple point mutations in the highly conserved hairpin loop iron response element region of the L-ferritin gene, which affect the interaction of iron regulatory protein with the iron response element(Girelli et al., 1997; Mikulits et al., 1999). Hence, the synthesis of L-ferritin is constitutive and poorly regulated. A surprising phenotype associated with this disorder is bilateral nuclear cataracts. Nevertheless, a direct relationship of the deposition of ferritin in the lens and mutation of the L ferritin iron response element has not been demonstrated (Cazzola et al., 1997).

I. Disorders related to mitochondrial iron homeostasis

The mitochondrion requires tightly regulated iron transport since it is the site of heme and iron-sulfur cluster synthesis, (Lange et al., 1999; Lill et al., 1999; Tong and Rouault, 2000). Recent discoveries in several diseases with mitochondrial pathology have allowed researchers to identify genes involved in mitochondrial iron homeostasis.

a. <u>Friedreich's ataxia</u>

One of the genes involved in regulation of iron efflux in the mitochondria is frataxin (Campuzano et al., 1997; Campuzano et al., 1996; Radisky et al., 1999). In patients with an autosomal recessive neurodegenerative disorder called Friedreich's
ataxia, there is an expansion of the GAA trinucleotides in the first intron of the frataxin gene, which results in a non functional protein (Gacy et al., 1998). Data from the yeast frataxin orthologue knockout models as well as histological and biochemical data from heart biopsies or autopsies of Friedreich's ataxia patients have shown that the frataxin defect causes a specific iron-sulfur protein deficiency and intra-mitochondrial iron accumulation (Babcock et al., 1997; Rotig et al., 1997). The increased mitochondrial iron accumulation results in increased oxidative stress and respiratory deficiency in mitochondria, which ultimately leads to irreversible mitochondrial damage (Babcock et al., 1997; Delatycki et al., 2000; Koutnikova et al., 1997; Wilson and Roof, 1997). However, a second hypothesis suggests that the time-dependent intra-mitochondrial iron accumulation in a frataxin-deficient mammal occurs only after the onset of the pathology (Puccio et al., 2001). This hypothesis implies that mitochondrial iron accumulation is the result of Friedreich's ataxia instead of the cause. Nevertheless, the use of iron chelating reagent, desferroxamine, is effective in both decreasing the iron deposition and inhibiting the progression of neurological symptoms (Palau, 2001; Wong et al., 1999).

b. <u>X-linked sideroblastic anemia</u>

Another gene associated with human mitochondrial iron homeostasis is the *abc7* gene, which encodes an ATP-binding cassette transporter (Kispal et al., 1997; Leighton and Schatz, 1995; Senbongi et al., 1999). A mutation in the human *ABC7* gene is responsible for the X-linked sideroblastic anemia ataxia, a non-progressive cerebella ataxia, which manifested in infancy or early childhood. (Allikmets et al., 1999). This gene has an orthologue in yeast called *atm1* (Csere et al., 1998). Previous studies have

shown that ATM1P transports the precursor of the mitochondrial iron-sulfur cluster into the cytosol (Kispal et al., 1997). Mutations in the yeast *atm1* gene result in iron accumulation in the mitochondria leading to oxidative damage (Senbongi et al., 1999).

IV. p97 and the transferrin family

The transferrin family is made up of serum transferrin, ovotransferrin, lactoferrin and p97 (also known as melanotransferrin). All members of the family consist of a single polypeptide chain of 80 kDa molecular weight and the crystal structures of several members of the transferrin family have been determined (Bailey et al., 1988; Baker and Lindley, 1992; Kurokawa et al., 1995; Smith et al., 1992). The polypeptide forms two very similar but distinct lobes, each capable of binding one atom of ferric iron together with a synergistic anion such as carbonate or bicarbonate anion. Amino acid sequence alignment of members of the transferrin family shows that they have extensive homology, especially in the areas where amino acids are involved in coordinating the iron atom (with the exception of p97) (Fig. 1). A detailed alignment of all the known p97 orthologues with members of the transferrin family is shown in Figure 1A.

A. Brief overview of serum transferrin, lactoferrin and ovotransferrin

Human serum transferrin was first identified by Schade and Caroline from the non-hemoglobin fraction of blood plasma in 1946 (Schade and Caroline, 1946). The primary structure of the protein revealed two highly homologous halves with 40% protein sequence identity, which are bridged by a connecting peptide (Fig. 2). Crystallographic studies of rabbit serum transferrin have revealed that the ferric ion is coordinated by the

Figure 1: The protein sequence alignment of members of the transferrin family including p97 from different species, human serum transferrin (Tf), human lactoferrin (LTf) and chicken ovotransferrin (OTf). (A) The complete protein sequence alignment of members of the transferrin family. The red colored amino acids are conserved residues whereas the blue colored amino acids are semi-conserved. \bigcirc denotes the amino acid residues involve in iron coordination and * denotes the amino acids involve in synergistic anions (such as carbonate and bicarbonate) coordination. $\bullet \bullet \bullet \bullet$ are the five amino acids involve in putative zinc binding as proposed by Garrett *et al.*(Garratt and Jhoti, 1992) and $\bullet \bullet \bullet$ denotes the integrin binding RGD motif. (B) Sketch diagram of the amino acids involved iron binding of the members of the transferrin family and the proposed zinc-binding site in different orthologues of p97.

102 nemr	abbit p97	ouse p97	nicken p97	ıman Tf	ıman LTf	iicken OTf	L <u>y</u> P97-1	LY P97-2
Hum	Rab	Mou	Chi	Hum	Hum	chi	F1Y	F1Y

nan p97	use p97	nan Tf	nan LTT	Y P97-1
obit p97	icken p97		icken OTf	V P97-2
Huma	Mous	Huma	Huma	Fly
Rabb	Chic		Chic	Fly

46	AGIQPSLLCVRGTSADHCVQLIAAQEADAITLDGGAIYEAG-KEHGLKPVVGEVYDQEVG
Ч	EADAITLDGGAIYEAG-KEHGLKPVVGEVYDQEVG
46	AGIRPSLLCVQGNSADHCVQLIKEQKADAITLDGGAIYEAG-KEHGLKPVVGEVYDQDIG
45	AGILPPLECMEGESAANCTQMIKDYLADTVTLDGRWIYQAG-KEYGLKPVVGEVYDQEIG
Ч	LADAVTLDAGLVYDAYLAPNNLKPVVAEFYGSKED
48	KVRGPPVSCIKRDSPIQCIQAIAENRADAVTLDGGFIYEAGLAPYKLRPVAAEVYGTERQ
47	QQERISLTCVQKATYLDCIKAIANNEADAISLDGGQAFEAGLAPYKLKPIAAEVYEHTEC
61	DEVFLNLTCFMAYSADECIHHIDREKAHITTLDAGDVFTAGRYNSLIPIMQEKLEC
53	LEAKSNIECVIGVDRLDCVRRIHKGTAHFGVLTSEDLVAAR WASVEILVASELRSHES

HFEYEIVAVVDNHANIHTVHDLRGARL CHPGYGLGNHWTEVLANYFEAAMVSKTCDP	111
GFADYQSVAVIKKGSLPDLNNLRDMRNKRVCFPWVGSLAGWIVPIHTLQREGGMEVVDC-	117
STTSYYAVAVVKKGTEFTVNDLQGKTS CHTGLGRSAGWNIPIGTLLHRGAIEWEGI -	107
PRTHYYAVAVVKKGGSFQLNELQGLKS CHTGLRRTAGWNVPTGTLRPFLNWTGP	108
PQTFYYAVAVVKKDSGFQMNQLRGKKS CHTGLGRSAGWNIPIG LLYCDLPEP	40
TSYYAVAVVRKGSNITINSLKGVRSCHTGINRTAGWNVPVGYLIDSGRLPAMG	104
TSYYAVAVVRRNSNVTINTLKGVKS CHTGINRTVGWNVPVGYLVESGHLSVMG	105
TSYYAVAVVKRSSNVTINTLRGVKS CHTGINRTVGWNVPVGYLVDSGRLSVMG	35
TSYYAVAVVRRSSHVTIDTLKGVKS CHTGINRTVGWNVPVGYLVESGRLSVMG	105

CDLPKAVSDYFSASCVPGTNSASYPTSLCQLCKGDSSGQNKCQGNS RKPLEKAVANFFSGSCAPCADGT-DFPQLCQLCPGCGCST PEPIEAAVARFFSASCVPGADKG-QFPNLCRLCAGT-GENKCAFSS ESGSVEQAVAKFFSASCVPGATG-QFPNLCRLCAGT-GENKCAR NNQVKTAASYFNNSCAVYSLSDKHNPIGDNSDKLCTLCTGKIPGGRCSS EMTVTEDRIASTAKYFGPSCKAGPWVPDPKQDRILKNRYPSLCEMCYEPDSCDQ	157 162 162 163 163 168
PEPIEAAVARFFSASCVPGADKG-OFPNLCRLCAGTGENKCAFSS	162
RKPLEKAVANFFSGSCAPCADGC-CGCST	92
CDLPKAVSDYFSASCVPGTNSASYPTSLCQLCKGDSSGQNKCQGNS	157
CDVLKAVGDYFGGSCVPGTGETSHSESLCRLCRGDSSGHNVCDKSP	158
CDVLKAVSEYFGGSCVPGAGETRYSESLCRLCRGDTSGEGVCDKSP	88
CDVLKAVSDYFGGSCVPGAGETSYSESLCRLCRGDSSGEGVCDKSP	158

Mouse p97 Chicken p97 Human Tf Human LTf

Human p97 Rabbit p97 Chicken OTf

Fly P97-1 Fly P97-2

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Iuman p97	auput p9/	chicken p97	Human Tf	luman LTf	Chicken OTf	'ly P97-1	'ly P97-2
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Human P97 Rabbit P97 Mouse P97 Chicken P97 Human LTf Human LTf Chicken OTf FlY P97-1 FlY P97-2 Human P97 Rabbit P97 Mouse P97 Chicken P97 Human Tf Human LTf Chicken OTf FlY P97-1 FlY P97-2

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DVGAQFQ 	304 226 301 296 340 319
H4CSMTG	296
DKSPKFQ	301
DKSKEF0	226
<mark>DVGAQFOI</mark>	304
0.2.2.2.2.2.2.2.2.2.2.2.2.2.2.2.2.2.2.2	305
0.1028BH	235
	305

Mouse p97 Chicken p97

Human p97 Rabbit p97 Chicken OTf

Fly P97-1 Fly P97-2

Human Tf Human LTf

244 SSEAYGQKNLLFKDSTLELVPIATQTYEAWLGP 314 SSKAYSQKNLLFKDSTLELVPIATQNYEAWLGQ 313 DSTAYGAONLMFRDSTTKLVAVTSONYOAWLGD

Mouse p97 Chicken p97 Human Tf Human LTf

Human p97 Rabbit p97 Chicken OTf

Fly P97 Fly P97-2

Human p97	364	PYLRWCVLSTPEIQKCGDMAVAFRRQRLKPEIQCVSAKSPQHCMERIQAEQVDAVTLSGE
Rabbit p97	294	PYLRWCVLSTPEIQKCGDMAVAFSRQRLKPEIQCVSAESPQHCMEQIQAGHIDAVTLNGE
Mouse p97	364	HYLRWCVLSAPEIQKCGDMAVAFSRQNLKPEIQCVSAESPEHCMEQIQAGHTDAVTLRGE
Chicken p97	363	ESLNWCVVSTEEIWKCGEMGTAFRSKNLKPEIQCISAKTKEECMEMIQKKEIDVVALGGV
Human Tf	289	KPVKWCALSHHERLKCDEWSVNSVGKIECVSAETTEDCIAKIMNGEADAMSLDGG
Human LTf	363	ARVVWCAVGEQELRKCNQWSGLSEG SVTCSSASTTEDCIALVLKGEADAMSLDGG
Chicken OTf	362	NRIQWCAVGKDEKSKCDRWSVVSNGDVECTVVDETKDCIIKIMKGEADAVALDGG
Fly P97-1	448	PAMTLCVTSENELDKCIKMRTALKAHLLKPELICKKMHSHINCMQFIEAGKADISVFDAG
Fly P97-2	368	RSIVFCTTSIIQHIKCSWLQEASQVYGVQPNIQCVRTMDEQQCLDNTKFKETDVVLVDQE

Chicken OTf Fly P97-1 Fly P97-2 Chicken p97 Human Tf Rabbit p97 Mouse p97 Human LTf Human p97

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424	DIYTAGKKYGLVPAAGEHYAPED SSNSYYVVAVVRDSSHAFTLDELRG
405 407	DIALAGKI YGLI PAAGELYAAUD NAVVKUDAYAFI VDELKG DIVDAGYYYYCI IIDAAGELYAED D GNEVERIAAD DD GOYGETTI DEI DG
423	DIYIAGKTYGLVPAAGESFSAED
344	FVYIAGKCG-LVPVLAENYNKSDNCEDTPEAGYFAVAVVKKS-ASDLTWDNLKG
418	YVYTACKCG-LVPVLAENYKSQQSSDPDPNCVDRPVEGYLAVAVVRRS-DTSLTMNSVKG
417	LVYTAGVCG-LVPVMAERYDDESQCSKTDERPASYFAVAVARKDSNVNWNNLKG
508	DVYTGGLNYDLVPFMSEVYNLGE DPDTELTYLKG
428	MRVKAQRDYNLVPLLYEFAADMHAKFESFRDLKG

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ARACLPSFEGAAHLSVQETIVNATGKVQSLHSYFHRDSCLWNLQSG	474
KNTCHTGINTAAGWTYPMALFISNGWIRPYGCDSVRAAAEYFTKSCVPGAISNEYNTGVP	554
KKSCHTAVGRTAGWVIPMGLIHNRGTGTCNFDEYFSEGCAPGSPPN	470
KKSCHTAVDRTAGWNIPMGLLFNQGCSCKFDEYFSQSCAPGSDPR	476
KKSCHTAVGRTAGWNIPMGLLYNKSINHCRFDEFFSEGCAPGSKKD	396
KKSCHTGLGRTAGWNIPIGMLVKKGFINPRDCNIPQAVSEFFSASCVPSAEQGNY	472
KRSCHPYLGSPAGWEVPIGSLIQRGFIRPKDCDVLTAVSQFFNASCVPVNNPKNY	473
KRSCHPGFGSPAGWDVPVGALIHWGYIRPRNCDVLTAVGQFFNASCVPVNNPKKY	403
KRSCHAGFGSPAGWDVPVGALIQRGFIRPKDCDVLTAVSEFFNASCVPVNNPKNY	473

Chicken p97

Mouse p97

Rabbit p97

Human p97

Chicken OTf

Human LTf

Human Tf

Fly P97-1 Fly P97-2

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RKCPLHYQGDEGALRCLSE-GADVAFLSSDVYKKYVVGNL	520
YDSMCDLCHG TSYRYCRRDASEEYYGHTGAFRCLVEGGGHVAFMKHTTVMESTGGKR	614
- SRLCQLCQGSGGIPPEKCVASSHEKYFGYTGALRCLVE - KGDVAFIQHSTVEENTGGKN	515
- SNLCALCIGDE - QGENKCVPNSNERYYGYTGAFRCLAENAGDVAFVKDVTVLQNTDGNN	521
- SSLCKLCMG SGLNLCEPNNKEGYYGYTGAFRCLVE - KGDVAFVKHQTVPQNTGGKN	441
PSTLCQLCIGDN-NGNNKCSASSQERYYSYNGAFRCLAEDAGDVAFVKHSTVFENTDGKN	527
PSALCALCVGDE-KGRNKCVGSSQERYYGYSGAFRCLVEHAGDVAFVKHTTVFENTNGHN	528
PSSLCALCVGDE-QGRNKCTGNSQERYYGDSGAFRCLVEGAGDVAFVKHTTIFDNTNGHN	458
PSSLCALCVGDE-QGRNKCVGNSQERYYGYRGAFRCLVENAGDVAFVRHTTVFDNTNGHN	528

Human p97 Rabbit p97 Mouse p97 Chicken p97 Chicken OTf Fly P97-1 Fly P97-2

Human Tf Human LTf

KADWAKNLQMDDFELLCTDGRRANVMDYRECNLAEVPTHAVVVRPEK-----ANKIRDL TSNWLTPGNHKDFRVLCPYGGIEKRSNFEYCYLHWTTRGHLMTHNSSLTR - - - RNEIYNS - IFTVYGL -- IFTVYGL ---IFALYGL --VERLKOV KEWWARNALNDDFELLCTDGTRAEIQDYKRCNLGKVKANAVVTRGGVNYNETQMNAYINL -- EACVHKI - IFTVYGL PEPWAAHLRSQDYELLCPNGARAEAHQFAACNLAQIPSHAVMVRPDTN--TESWARDLKSSGFQLLCRNGARAEVTQFAQCHLARVPAQAIMVHPDTN - -PDPWAKNLNEKDYELLCLDGTRKPVEEYANCHLARAPNHAVVTRKDK - --PEPWASHLRWQDYELLCPNGARAEVDQFQACNLAQMPSHAVMVRPDTN--SEPWAAELRSEDYELLCPNGARAEVSQFAACNLAQIPPHAVMVRPDTN-NEAWAKDLKLADFALLCLDGKRKPVTEARSCHLAMAPNHAVVSRMDK-587 517 587 586 496 579 573 671 559

Chicken p97

Mouse p97

Rabbit p97

Human p97

Chicken OTf

Human Lf

Human Tf

Fly P97-1 Fly P97-2

LTYAQQLYGRKEVDAFS-FSMFSSPIG-HYDLIFQDATRQLQVIPPNKRYDAYLGSDFM LDKAQDLFGDDHNKNG - - FKMFDSSSYHGRDLLFKDATVRAVPVG - ERTTYQDMLGPDYV LLHQQAKFGRNGSDCPDKFCLFQSETK- - - NLLFNDNTECLARLH-GKTTYEKYLGPQYV LRDMDQLFGRKYKSETRPFTLYGIFDK-RNNVLFRDDTDGLLGLQELHRDNAKRVMEHIY LDKAQDLFGDDHNKNG - - FQMFDSSKYHSQDLLFKDATVRAVPVR - EKTTYLDWLGPDYV LDKAQEYFGNNSNRNG - - FKMFDSSAFQGKDLIFKDSAVKIVPVE - ERRTYAEWLGSEYV LRQQQHLFGSNVTDCSGNFCLFRSETK - - - DLLFRDDTVCLAKLH - DRNTYEKYLGEEYV LERQEKRFGUNGSEKS - KFMMFESQNK - - - DLLFKDLTKCLFKVR - EGTTYKEFLGDKFY LDKAQDLFGDDHNKNG--FKMFDSSNYHGQDLLFKDATVRAVPVG-EKTTYRGWLGLDYV 642 572 642 641 550 633 627 731 616 Chicken p97 Chicken OTf Rabbit p97 Mouse p97 Fly P97-1 Fly P97-2 Human p97 Human Lf Human Tf

Human p97 Rabbit p97 Mouse p97	699 629 699	AALEGMSSQQCSGAAAPAPGAPLLPLLLPALAARLLPPAL- AALEGMQSQRCSGAAVGAPGASLLPLLPLAVGLLLSSL VALEGMLSQQCSGAGAAVQRVPLLALLLLTLAAGLLPRVL-
Chicken p97	698	ESLEGMQTPQCSGAGNKLIQQHLLVITFVPFIILGQLQGLG
Human Tf	606	KAVGNLRKCSTSSLLEACTFRRP
Human Lf	689	AGITNLKKCSTSPLLEACEFLRK
Chicken OTf	682	TVISSLKTCNPSDILQMCSFLEGK
Fly p97	789	RARITDCYAGASQLALSVGLLLVGSLVAML
Fly P97-2	675	DRYANTQYYRNFDESGAMKSSHNLWILLVCLLFVVGQPFG-



(B)



Transferrin

p97

Figure 2: Sketch of the bilobed structure of transferrin and p97. Aspartic acid (D), tyrosine (Y) and histidine (H) are important amino acid residues involved in coordination of iron atom (Fe3+) in the binding site. The aspartic acid in the C-lobe of p97 is changed to a serine (S) residue.

carboxylate oxygen of D82, two phenolate oxygens from Y114 and Y207 and the imidazole nitrogen of H268. The hydrogen bonding network to the synergistic carbonate anion is provided by T139, R143 and S144 (Baker and Lindley, 1992).

The uptake of iron by transferrin/ transferrin receptor is mediated by clathrinmediated pathway (Fig. 3). Briefly, circulating holo-transferrin binds to the transferrin receptor on the cell surface at physiological pH. Once bound, the transferrin/ transferrin receptor complex is endocytosed into the cell through the formation of the clathrin-coated pit. The clathrin-coated vesicles either mature into or fuse with endosomes. The decrease in intra-vesicular pH results in a decrease in the affinity of iron for transferrin, causing iron to be released from the transferrin/ transferrin receptor complex. The iron is transported out of the endosomal compartment by the divalent metal transporter Nramp2 and into the cytoplasm. The cytoplasmic iron is transferred either to ferritin for storage or to other organelles such as mitochondria for cellular usage (Fleming et al., 1998; Tabuchi et al., 2000).

The interaction between transferrin receptor and the hemochromatosis protein, HFE, has required the re-evaluation of iron uptake by this pathway. *In vivo* studies show that holo-transferrin, transferrin receptor and HFE interact in a 1:2:1 ratio (Gross et al., 1998; Lebron et al., 1998). HFE complexes with transferrin receptor in the endoplasmic reticulum and the complex traffics to the plasma membrane via the Golgi network. The association of HFE and transferrin receptor is required for locating transferrin receptor on the cell surface (Gross et al., 1998; Salter-Cid et al., 2000a) and for decreasing the rate of endocytosis of transferrin/ transferrin receptor complex, which results in a downregulation of transferrin-dependent iron uptake (Corsi et al., 1999;

Figure 3: Iron uptake mediated by transferrin/ transferrin receptor. Circulating holotransferrin binds to the cells surface transferrin receptor-HFE complex at physiological pH and the complex is endocytosed through formation of the clathrin-coated pit. The clathrin-coated vesicles mature into or fuse with endosomes. The decrease in intravesicular to approximately pH 5.5 results in the decrease in the affinity of iron for transferrin and hence iron (Fe3+) is released from the complex. Early endosomes gradually matures into late endosomes in the endocytic pathway. The iron is transported out of the late endosomes or lysosomes by the divalent metal transporter, Nramp2, into the cytoplasm, which is either incorporated into ferritin for storage or transferred to other organelles such as mitochondria for cellular usage



Riedel et al., 1999; Roy et al., 1999). Hence, HFE, through its binding to the transferrin receptor, appears to function as a negative regulator of transferrin receptor mediated uptake of iron.

Lactoferrin shares a 40% protein sequence homology with human serum transferrin. It is an 80 kDa glycoprotein secreted mainly by the glandular epithelial cell of the mammary gland (Campbell et al., 1992). It is also present in the secretion of other exocrine fluids such as saliva, bile, pancreatic fluid and tears (Colomb et al., 1976; Inoue et al., 1993; Janssen and van Bijsterveld, 1983; Jonas et al., 1993; Saito and Nakanuma, 1992). Lactoferrin appears to have additional functions other than iron-binding. Lactoferrin is stored in the secondary granules of polymorphonuclear neutrophils and is released during inflammation (Crouch et al., 1992). It modulates the inflammatory response by blocking the formation of C3 convertase in the classical complement pathway (Britigan et al., 1994; Rainard, 1993). In addition, lactoferrin promotes the rapid migration of polymorphonuclear neutrophils from blood to the site of inflammation by increasing P-selectin mediated binding of the leukocytes to the endothelium (Asako et al., 1994; Kurose et al., 1994). Lactoferrin also plays a role in host defense at mucosal surfaces due to its antibacterial properties (Haversen et al., 2000). In such a role, lactoferrin functions as an iron chelator, exerting a bacteriostatic effect by denying the bacteria access to iron. (Spik and Montreuil, 1983). Another immune modulation effect exerted by lactoferrin is its ability to reduce the secretion of IL-1, IL-6 and tumor necrosis factor- α from monocytes in response to bacterial lipopolysaccaride (Crouch et al., 1992; Cumberbatch et al., 2000; Otsuki et al., 1998).

Ovotransferrin is one of the components of the egg white protein and is implicated in the transfer of trace elements from the hen oviduct into developing chicken embryos. The high level of amino acid sequence homology between ovotransferrin and the rest of the transferrin family members suggests that they share similar biological roles in binding and transporting irons. However, recently the anti-microbial properties of ovotransferrin have been investigated. A cationic fragment of ovotransferrin appears to interact with bacterial membranes and cause damage to the bacterial cytoplasmic membranes (Ibrahim et al., 1998; Ibrahim et al., 2000).

B. <u>Overview of p97</u>

a. <u>p97 as an iron-binding protein</u>

P97, also known as melanotransferrin, is a 97 kDa protein, which shares a 40% protein sequence identity with human lactoferrin (Rose et al., 1986). It was first identified as a cell surface marker for human skin cancer (Brown et al., 1981; Woodbury et al., 1980). However, the protein was subsequently found expressed at various levels in liver, intestine, umbilical cord, placenta, sweat gland and more recently in human brain endothelium (Barresi and Tuccari, 1994; Rothenberger et al., 1996; Sciot et al., 1989). The localization of p97 to the capillary endothelium in the brain led to the proposal that p97 may play a role in iron transport to the brain (Rothenberger et al., 1996). Immunohistochemistry and immune electron microscopy studies have also localized p97 to the apical brush border of epithelial cells in the fetal intestine (Alemany et al., 1993).

Unlike the other members of the transferrin family, p97 has two different forms. It can be found as a membrane protein attached to the cell surface via a glycosylphosphatidylinositol (GPI) anchor or as a soluble form in serum (Alemany et al., 1993; Brown and Rose, 1992; Food, 1992). These forms are thought to originate from alternative splicing of the p97 mRNA (Hsu, 1997; McNagny et al., 1996). Metal binding studies on a cell line transfected with human p97 show GPI-anchored p97 can mediate iron uptake where the process is both temperature dependent and saturable (Kennard et al., 1995). Like other members of the transferrin family, p97 is a bi-lobed metal binding protein (Fig. 2). Protein sequence alignment of p97 with other members of the transferrin family shows that the amino acids involved in coordinating the iron atom in the N-lobe are totally conserved (Rose et al., 1986) (Fig. 1 and 2). However, there are at least four specific amino acid substitutions in the C-lobe of p97 which were thought to affect the ability of the protein to bind iron (Fig. 1): (i) The aspartic acid at position 421 is changed to a serine residue (D421S). The D421S substitution is thought to affect the simultaneous hydrogen bonding across the iron binding cleft and results in a failure to lock in the iron atom when it is bound. (ii) The arginine at 482 is changed to a serine residue (R482S). (iii) Threonine at position 478 is changed to an alanine residue (T478A). (iv) Threonine at position 483 is changed to a proline residue (T483P). Both R482S and T478A substitutions are thought to affect the binding of carbonate anion of the binding site and the T483P substitution is proposed to disrupt the hydrogen bond between threonine and aspartic residue at 421. Nevertheless, the crystal structure of human lactoferrin with a D60S amino acid substitution in the N-lobe, which mirrors the D421S substitution in p97, shows that a water molecule coordinate the iron atom and allows the iron to bind to the

site. The binding of iron to the modified pocket in this situation may be weakened (Faber et al., 1996).

b. <u>Regulation of p97 expression</u>

The binding of the iron regulatory protein to the iron response element is an exquisite system used by cells to regulate the expression of iron proteins involved in maintaining the delicate balance of iron homeostasis (Cairo and Pietrangelo, 2000; Haile, 1999). Most but not all proteins involved in iron metabolism have a conserved RNA stem-loop structure located either on the 3 or 5 untranslated region of the mRNA called the iron response element. When iron is abundant, the iron regulatory protein does not bind to the iron response element. However, when iron is scarce, the iron regulatory protein binds to the iron response element and confers translational regulation. If the iron response element is located in the 3 untranslated region of the mRNA (such as that of transferrin receptor mRNA), binding of iron regulatory protein to the iron response element of the mRNA stabilizes the transcript and thereby enhances the translation of the protein. However, if the iron response element is located in the 5 untranslated region of the transcript (for example, ferritin mRNA), the binding of the iron regulatory protein to the iron response element disrupts translation (for review please see Eisenstein, 2000). In contrast to the transferrin receptor and ferritin, where the level of protein expression is regulated by the level of iron through the iron response element/ iron regulatory protein system, there is no identifiable iron response element motif in p97 and the level of expression is not regulated by iron (Richardson, 2000). However, a regulatory element has been located 2kb upstream of the p97 promoter region. Deletion of this element

severely impairs the expression of p97 (Duchange et al., 1992). This regulatory element was shown to be part of an enhancer composed of two binding sites for AP-1 transcription factor. AP-1 is a transcription factor that is up-regulated after ultra-violet irradiation, a suspected risk factor in melanoma incidence. This may explain how p97 is up-regulated in melanoma cells (Derijard et al., 1994; Devary et al., 1991).

c. Orthologues of p97

Several orthologues of p97 have been identified and cloned (Fig. 1). The protein sequences of these different p97s show a much higher sequence homology between themselves compared to other transferrin family members. The conservation of all 28 cysteine residues in all the various p97 suggests similar protein folding and tertiary structure with the other transferrins. The identification of the different p97 orthologues has implied a variety of functions for the molecules.

The chicken p97 (EOS 47) has a 61% protein sequence identity with human p97 (McNagny et al., 1996). Unlike other p97 orthologues that have conserved iron-binding residues in the N-lobe and non-conserved iron-binding residues in the C-lobe, the chicken p97 has an additional H278R amino acid substitution that may prevent iron-binding in the N-lobe. Hence, chicken p97, with both atypical N-and C-lobes, appears to have evolved away from high affinity iron-binding. Chicken p97 is highly expressed in epithelial brush borders of small intestine and kidney and expressed at lower levels in cells lining the sinusoids of the liver. A sub-population of eosinophils in the chicken bone marrow also expresses increased level of chicken p97, however, it is undetectable in the matured peripheral eosinophils, suggesting that the expression of chicken p97 ceases shortly

before these cells emigrate from the bone marrow into the peripheral blood. In addition, chicken p97 appears to be a direct target of the GATA-1 and C/EBP β transcription factors, which are responsible for the differentiation of eosinophils (McNagny et al., 1996). In this regard, chicken p97 seems to be a developmentally regulated during eosinophil maturation.

Following the cloning of chicken p97, rabbit p97 was cloned from rabbit chondrocyte cultures. The protein sequence of rabbit p97 shows 85% identity to human p97. Western blot, Northern blot and reverse-transcription PCR analyses indicated that rabbit p97 is highly expressed in cartilage and chondrocytes. Immunohistochemistry staining of the rabbit chrondrocytes demonstrated that rabbit p97 is expressed on the plasma membrane of the cells. Rabbit p97 transcripts were detected in all zones of the cartilage but the level was relatively low in the hypertrophic zone. These findings suggest that p97 is involved in maintaining the cell surface characteristics of chondrocytes (Kawamoto et al., 1998).

The mouse orthologue of p97 was cloned from a mouse embryonic cell line and it shows 62% protein sequence homology to the human p97 (Nakamasu et al., 1999). Sequence alignment shows that the amino acids involved in iron coordination in the Nlobe is conserved, whereas the C-lobe shows a D421R and R482S amino acid substitutions in the iron-binding pocket. It is suggested that the amino acid substitutions can impair iron binding (Nakamasu et al., 1999). Northern blot analysis shows that tissue expression pattern of mouse p97 is different from human p97. While human p97 is widely expressed, the expression of mouse p97 seems to be restricted to testes and cartilage tissues and its expression seems to be regulated during chondrocyte differentiation (Nakamasu et al., 1999).

The completion of the *Drosophila melanogaster* genome sequence allowed the identification of two fly orthologues of p97, fly p97-1 and fly p97-2. The absence of proteins orthologous to vertebrate transferrin receptor in the fly would suggest that the fly orthologues of p97 could mediate the insect pathway for cellular uptake of iron and other metal ligands (Adams et al., 2000). However, some of the critical iron-binding residues in both the fly homologues are not conserved. Therefore, fly p97 might play a role separate from its ability to bind iron.

d. <u>Alzheimer s disease and p97</u>

Expression of p97 has been examined in brains from Alzheimer s disease patients Immunohistochemical staining shows that p97 is highly expressed in a subset of reactive microglial cells associated with β -amyloid positive senile plaques in Alzheimer s disease brains (Jefferies et al., 1996). This has been confirmed by positive *in situ* hybridization of p97 mRNA in reactive microglial cells in Alzheimer s disease brain sections (Yamada et al., 1999). Soluble p97 was also found to be elevated in the cerebral spinal fluid and serum of patients with Alzheimer s disease compared with healthy age matched controls (Kennard et al., 1996; Kim et al., 2001). In addition, serum p97 has been shown to cross the blood-brain barrier and deliver iron across the blood-brain barrier more efficiently than transferrin, if both are injected into the tail vein of the mouse (Moroo et al., 2001). Hence, it was proposed that p97 could play a role in the pathology of Alzheimer s disease. Metals such as iron, zinc and copper have been shown to precipitate β -amyloid

plaque formation (Bush, 2000; Bush et al., 1994). P97 could be involved in the disease by transporting iron or other metals across the blood-brain barrier, which could augment amyloid plaque formation. However, the mechanism of p97 transport across the blood-brain barrier is still being investigated. Alternatively, expression of p97 on reactive microglial cells could be a result of inflammatory response related to Alzheimer s disease. p97 may bind excess amounts of free iron released from damaged tissues and therefore provide a protective effect against iron generated free radicals. Nevertheless, these two hypotheses are still being tested.

e. <u>Other possible functions of p97</u>

The identification of several serum transferrin homologues such as saxiphilin in bullfrog, pacifastin in crayfish and porcine inhibitor of carbonic anhydrase in pig, which have physiological roles unrelated to iron transport, leads to the proposal that p97 might have alternate functions (Lenarcic et al., 2000; Liang et al., 1997; Wuebbens et al., 1997). The presence of a proposed zinc-binding motif (³⁴⁶ HEYLH ³⁵⁰) between the N- and the C-lobes, which is identical to the consensus Zn-binding motif in thermolysin, suggests that p97 might have metalloprotease activity (Garratt and Jhoti, 1992). However, the **HEXXH** motif was not conserved in other p97 orthologues (Fig. 1B). Therefore, the significance of human p97 possessing the zinc binding activity while p97s from other species do not, is in doubt.

A recent review proposes a key and lock interaction between GPI-anchored p97 on one cell and transferrin receptor on another cell (Sekyere and Richardson, 2000). It was suggested that the interaction would allow docking of p97 on the cell surface and

thus target the metalloprotease action to the site. Recently it was also suggested that p97 could function as an intercellular adhesion molecule (Sekyere and Richardson, 2000). Sequence analysis of the human and mouse p97 shows that the human protein has one arginine-glycine-aspartic acid (RGD) motif whereas the mouse p97 has two. RGD motif is proposed to serve as an attachment site for integrin during cell-cell adhesion (Ruoslahti, 1996). Having a RGD motif could imply that p97 plays a role in cellular adhesion by interacting with cell surface integrin molecules (Ruoslahti, 1996). Nevertheless, there is no experimental data to support this claim.

While it has been shown that GPI-anchored p97 can mediate cellular iron uptake, the function of soluble p97 has not been defined. There are data indicating that soluble p97 can cross the blood-brain barrier, however, the identity of the receptor that mediates the transcytosis of soluble p97 is still under investigation (Moroo et al., 2001). There is a preliminary report indicating that soluble p97 can bind to transferrin receptor (Walker et al., 1999) but obviously, further investigations are required to define both the metalloprotease activity and the intercellular adhesion mechanism of p97.

V. Transferrin/ transferrin receptor independent iron transport

The existence of transferrin/ transferrin receptor-independent iron transport pathways is shown from evidence that transferrin is not universally required for efficient iron uptake. For example, hypotransferrinemic (hpx) mice have massive tissue iron overload in all non-hematopoietic tissues even though serum transferrin levels are low (Bernstein, 1987; Craven et al., 1987). The liver iron burden in these animals is

100-fold higher than that of normal wild-type mice (Trenor et al., 2000). Clearly, transferrin-independent mechanisms are mediating the iron transport in these mice.

There are three general categories of transferrin/ transferrin receptor independent iron transport: (A) Transferrin-dependent transferrin receptor-independent pathway (e.g. iron transport mediated by binding of transferrin to the newly identified transferrin receptor 2); (B) Transferrin-independent transferrin receptor-dependent pathway (e.g. the proposed binding of soluble p97 to transferrin receptor); (C) Transferrin and transferrin receptor-independent pathways (e.g. iron uptake mediated by GPI-anchored p97, iron absorption mediated by heme receptor, Nramp2 and paraferritin complex in the intestine).

A. <u>Transferrin-dependent transferrin receptor-independent pathway</u>

The homologue of transferrin receptor 1, transferrin receptor 2, has recently been identified. The human transferrin receptor 2 is a type II transmembrane glycoprotein that shares 66% protein sequence similarity in its extracellular domain with transferrin receptor (Kawabata et al., 2000; Kawabata et al., 1999). Kawabata *et al.* showed that transferrin receptor 2 could bind and internalize transferrin, even though transferrin has a lower affinity for transferrin receptor 2 (Kawabata et al., 2000; West et al., 2000). There are also controversies as to whether transferrin receptor 2 can associate with HFE like transferrin receptor 1 and whether HFE has any effect on the transferrin binding to transferrin receptor 2 (Lieu et al., 2001; West et al., 2000).

Unlike the ubiquitous expression of transferrin receptor 1 expression, transferrin receptor 2 is predominantly expressed in the liver. In addition, transferrin receptor 2

does not have the iron response element, which means that its expression is not regulated by cellular levels of iron. The lack of the iron regulatory element in transferrin receptor 2 may contribute to iron overloading due to uptake of transferrin bound iron despite down-regulation of transferrin receptor 1 expression by high levels of iron (Fleming et al., 2000). Clearly, further investigations are required to define whether transferrin receptor 1 and transferrin receptor 2 are regulated by distinct pathways and whether they mediate iron uptake by different mechanisms.

B. Transferrin-independent transferrin receptor-dependent pathway

The high homology of p97 with serum transferrin suggests that it could bind to the transferrin receptor or even transferrin receptor 2. Experiments have also shown that soluble p97 is able to cross the blood-brain barrier into the brain and it is likely that this process is receptor mediated (Moroo et al., 2001). Moreover, there is a preliminary report indicating that soluble p97 can bind to transferrin receptor (Walker et al., 1999). The high sequence homology between transferrin receptor and transferrin receptor 2 suggests that transferrin receptor 2 cannot be ruled out as a possible receptor for p97.

C. Transferrin/ transferrin receptor -independent pathways

Transferrin/ transferrin receptor independent pathways include examples of iron transport by GPI-anchored p97 and iron absorption in the intestine.

a. <u>GPI-anchored p97</u>

GPI-anchored p97 is able to transport iron in p97 transfected Chinese hamster ovary cell line and this process is both temperature dependent and saturable (Kennard et al., 1995). In SK-MEL 28 human melanoma cells line, where the level of p97 expression is elevated, a similar activity has been studied, which suggested that iron uptake can be mediated by p97 (Richardson and Baker, 1991a; Richardson and Baker, 1991b). It is interesting to note that GPI-anchored p97 is also found in the apical brush border of fetal intestine, which implies a possible function in gut iron absorption (Alemany et al., 1993).

b. <u>Iron absorption in the intestine</u>

Iron homeostasis is controlled by absorption rather than by excretion because there is no known specific iron excretion mechanism in higher organisms. These cells are responsible for sensing the internal iron load in the serum and responding appropriately by altering iron uptake using one of at least three pathways known to mediate iron absorption on the apical side of the absorptive villus cell (Fig. 4).

i. Ferrous iron absorption by Nramp2

The most extensively characterized iron absorption pathway is via a membrane spanning protein called Nramp2 (also known as the divalent metal transporter, DMT-1 or DCT1). Nramp2 is a metal-proton symporter that transports ferrous iron from the intestinal lumen into the villus cell (Canonne-Hergaux et al., 1999; Fleming et al., 1998; Gunshin et al., 1997). Nramp2 is capable of transporting not only ferrous iron but also a broad range of divalent cations including Zn^{2+} , Mn^{2+} , Co^{2+} , Cd^{2+} , Cu^{2+} , Ni^{2+} and Pb^{2+} (Gunshin et al., 1997). It is expressed on the apical plasma membrane of the villus as

Figure 4: The intestinal iron absorption pathways. The HFE/ transferrin/ transferrin receptor complex relays the level of plasma iron to the immature crypt cells. The level of iron regulates a concerted action of post-transcriptional regulatory system of iron response element and iron regulatory protein to modulate the expression of proteins involved in iron absorption in the mature villus cells. There are three proposed pathways for intestinal iron absorption. (1) Dietary iron in the ferric form (Fe3+) can be reduced to ferrous iron (Fe2+) by duodenal associated ferric reductase (Dcytb). (2) Fe2+ and Fe3+ can be absorbed by the villus cell by the paraferritin complex, consisting of β -integrin, mobilferrin and flavin mono-oxygenase. (3) Heme is released from myoglobin and hemoglobin in the intestinal lumen by gastric enzymes and enters the villus cell as intact metalloporphyrin through a heme receptor. Iron is released from heme by heme oxygenase-1 (HO-1) and transported across the vesicular membrane by iron dependent ATPase (Fe-ATPase). Intracellular iron can either be stored in ferritin or be transported across the basolateral membrane by IREG1. Hephaestin appears to facilitate iron egress by oxidizing Fe2+ into Fe3+, which is incorporated into serum apo-transferrin.



well as internally on the membrane of late endosomes and lysosomes of the cell (Fleming et al., 1998; Tabuchi et al., 2000). The apical Nramp2 protein mediates divalent metal absorption from the intestine whereas the vesicular Nramp2 is responsible for metal egress from the endosomes (Fleming et al., 1999; Griffiths et al., 2000; Tabuchi et al., 2000).

The expression of Nramp2 on the apical surface of intestinal villus cell is regulated by the level of labile iron pool in the cell (Andrews, 1999). Other proteins including the Iron-regulated transporter (IREG1)(also known as ferroportin in zebra fish) and the recently cloned apical ferric reductase, Dcytb, are also regulated by this level (Donovan et al., 2000; McKie et al., 2001). The level of labile iron pool is established in the enterocyte precursor called the crypt cell. In the crypt cell, transferrin receptor bound to the HFE at the basal lateral side of the cell facilitates the uptake of transferrin from the plasma. Iron from transferrin contributes to the labile iron pool inside the cell. This pool of labile iron modulates the activities of the iron regulatory proteins inside the crypt cell. If the level of labile iron pool is low, the translation of Nramp2, IREG1 and Dcytb is initiated when the crypt cell matures into absorptive villus cell. The apical ferric reductase, Dcytb, functions to reduce dietary ferric iron to ferrous form before the iron is transported by Nramp2. Once iron is transported into the villus cells, it has to exit the villus cell into the plasma. IREG1, a basal lateral membrane protein of the villus cell, functions to transport iron into the plasma. Once transported, hephaestin, a copper containing ferroxidase, localized to the basal lateral membrane of the villus cell, oxidizes the ferrous iron to ferric irons and promotes the release of iron to the circulating apotransferrin in the serum (McKie et al., 2000; Vulpe et al., 1999).

ii Paraferritin-mediated iron absorption

A second proposed pathway of iron absorption in the intestine is mediated by a 520 kDa protein complex called paraferritin, which is made up of β -integrin, mobilferrin and flavin monooxygenase (Conrad and Umbreit, 1993; Conrad et al., 1993). Ferric iron solubilized by mucin in the gut lumen, are bound to the cell surface β 3-integrin and transferred to mobilferrin, which is found docked on the inner cytoplasmic leaflet of the plasma membrane (Conrad et al., 1994). Flavin monooxygenase becomes associated with the β 3 integrin-mobilferrin complex and reduces the iron to ferrous form in concert with the oxidation of NADPH. The iron can then be incorporated into heme, ferritin or other molecules.

iii. <u>Heme absorption</u>

Heme is another important source of dietary iron (Ponka, 1999). Hemoglobinand myoglobin-derived iron are absorbed more efficiently by the intestine than inorganic iron because the presence of amino acids from proteolytic digestion helps to prevent polymerization and precipitation of heme (Majuri and Grasbeck, 1987; Raffin et al., 1974; Turnbull et al., 1989). The intact metalloporphyrin is liberated from the hemoglobin and myoglobin through enzymatic digestion in the intestinal lumen. There is evidence to suggest the presence of a heme receptor on the luminal side of villus cells that transports the metalloporphyrin form of heme into the cell (Grasbeck et al., 1982). Once heme is inside the cell, iron is released via heme oxygenase-1 (HO-1), which cleaves the heme ring to generate bilirubin, carbon monoxide, and ferrous iron (Maines,

1997). In mice deficient in HO-1, tissue iron stores are high while serum iron levels are low, suggesting that the enzyme is involved in the discharge of iron from certain cells (Poss and Tonegawa, 1997a; Poss and Tonegawa, 1997b). The method by which iron is liberated from heme and subsequently routed for use systemically is not fully understood. Recently, a mammalian iron ATPase (Fe-ATPase) associated with the microsomal membrane and co-distributed in tissues with HO-1 was identified (Baranano et al., 2000). There is some evidence to suggest that HO-1 is functionally coupled to the Fe-ATPase. This novel Fe-ATPase is enriched in spleen and mediates the iron transport upon hydrolyzing nucleotide triphosphate (Ferris et al., 1999; Poss and Tonegawa, 1997a). Bara ano and associates propose a model in which Fe-ATPase and HO-1 co-localize to the endoplasmic reticulum where heme is first degraded by HO-1 and the freed iron is transported by the Fe-ATPase to the luminal side of the endoplasmic reticulum (Baranano et al., 2000). They suggested that the iron in the endoplasmic reticulum could then bind to newly synthesized transferrin and subsequently be recycled back to the cell surface for exocytosis.

VI. Hypothesis and General approach

The focus of this thesis was to add to the basic understanding of the function of p97. The main hypothesis of this thesis was p97 is an iron binding protein that is actively involved in iron transport into cells. At the start of this research, there were questions regarding the validity of p97 as an iron transport protein for several reasons. First, although sequence analysis showed that p97 shared high sequence homology with other members of the transferrin family, only the amino acids involved in iron coordination in

the N-lobe were conserved. The C-lobe of human p97 was proposed not to bind to iron due to two amino acids substitutions at D421S and R482S, in contrast to other members of transferrin family where these amino acids were totally conserved (Fig. 1). The crystal structure of human lactoferrin, with a D60S substitution in the N-lobe that mirrors the D421S substitution in human p97, shows that iron can bind to the site. This suggests that the Asp to Ser substitution would not severely affect the ability of the protein to bind iron (Faber et al., 1996). Second, there were doubts as to whether any iron bound and transported by p97 could be donated to ferritin (Richardson and Baker, 1991a; Richardson and Baker, 1991b). Third, there was evidence showing that metals such as iron, copper, zinc and aluminum play important roles in several neurological disorders such as Alzheimer s disease and Parkinson s diseases (Bush, 2000; Bush et al., 1994; Perl and Pendlebury, 1986; Sayre et al., 2000). However, the route of metal uptake into the brain is not known. Soluble p97 has been shown to cross the blood-brain barrier (Moroo et al., 2001). Therefore, it was hypothesized that p97 could mediate the uptake of metal ions into the brain. However, before establishing whether p97 can mediate the transport if these metals across the blood-brain barrier *in vivo*, it is important to determine whether p97 can bind to these metals *in vitro*. It is known that transferrin can bind to other metals such as zinc, copper and aluminum at the same iron-binding site. Thus, p97, with identical iron-binding site in the N-lobe, might be able to bind to these metals as well.

The first results chapter therefore addresses whether the p97 can indeed bind two atoms of iron and relatedly whether p97 can bind other metals. Iron binding to p97 was addressed using UV-visible spectroscopy and differential scanning calorimetry. The binding constant of iron to p97 was ascertained using calorimetric method and the ability

of p97 to bind other metals was demonstrated through competition experiment in the presence of iron. Urea polyacrylamide gel analysis was used to corroborate the binding of other metals to p97. In addition, to resolve whether iron transported by p97 is delivered to ferritin, iron loading of ferritin was compared between p97 transfected and non-transfected cell lines.

The second results chapter of the thesis examines the endocytosis pathway of GPI-anchored p97 and follows the fate of the iron as it is being taken up by p97. Though the internalization pathways of many GPI-anchored proteins have been studied in detail, debate still exists on whether caveolae or clathrin-coated vesicles are involved in their uptake. For example, there are debates on whether GPI-anchored prion protein is localized to caveolae or clathrin coated pits (Harmey et al., 1995; Shyng et al., 1994). Furthermore, very little is known about the fate of ligands bound to GPI-anchored molecules after they are internalized. The most studied ligand internalized by a GPIanchored protein is the uptake of folate by GPI-anchored folate receptor (Anderson, 1998; Anderson et al., 1992). However, there are disagreements on whether the folate is trafficked to endosomes once it is internalized (Kamen et al., 1988; Rothberg et al., 1990). Confocal immunofluorescence microscopy and sub-cellular fractionation was used to address whether GPI-anchored p97 bound with iron is endocytosed into caveolae or clathrin-coated pits. Furthermore, the internalization pathway of GPI-anchored p97 in the cytoplasm was followed. GPI-anchored p97 was shown to traffic to the endosomes and subsequently to vesicles containing Nramp2. The observations led to a proposed model describing how iron is transported by GPI-anchored p97 into the vesicles and eventually donated to the ferritin in the cytoplasm.

Taken together, the studies on p97 provide new and fundamental information on both transferrin/ transferrin receptor independent uptake of iron and also on GPI anchor mediated uptake of ligands.
Chapter 2: Materials and Methods

I. Tissue culture, cells and antibodies

TRVb cell line, a Chinese Hamster Ovary (CHO) cell line that does not express functional endogenous hamster transferrin receptor, was obtained from Dr. F. Maxfield (New York University, NY) (McGraw et al., 1987). This cell line was co-transfected with pSV2p97a, encoding the full-length human p97 cDNA driven by the SV40 early promoter, and the geneticin (G418) resistance vector, pSV2neo (Food et al., 1994). The p97 transfected cell line is called p97⁺ TRVb in this thesis. TRVb transfected with human transferrin receptor clone pCDTR1 and pSV3neo, was also obtained from Dr. F Maxfield. The transferrin receptor positive cell line is named TfR⁺TRVb in this thesis. All three cell lines were maintained in Ham's-F12 media (Invitrogen Life Technologies Inc., Burlington, ON) supplemented with 10% (v/v) fetal bovine serum (Invitrogen Life Technologies Inc.), 2 mM glutamine (Invitrogen Life Technologies Inc.), 20 mM HEPES (Sigma-Aldrich Canada Ltd), 100 U/ml penicillin (Sigma-Aldrich Canada Ltd, Oakville ON) and 100 g/ml streptomycin (Sigma-Aldrich Canada Ltd), pH 7.4, at 37 °C in a 5% CO₂ humidified incubator. The media for $p97^{+}TRVb$ also included 500 µg/ml G418 (Invitrogen Life Technologies Inc.).

The human melanoma cell line SK-MEL 28 which expresses high levels of human p97, was obtained from American Tissue Type Culture Collection (ATCC, Manassas, VA). This cell line was maintained in DMEM supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine, 20 mM HEPES, 100 U/ml penicillin and 100 μ g/ml streptomycin, pH 7.4, at 37°C in a 5% CO₂ humidified incubator.

Antibodies used in this study are listed in Table IIIa and table IIIb.

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Antibodies	Antigen	Host	<u>Isotype</u>	Concentration	<u>Source</u>
L235	Human p97	Mouse	IgG1	15 mg/ml	ATCC
OKT-9	Human TfR	Mouse	IgG1	12 mg/ml	ATCC
Caveolin	Human caveolin-	Rabbit	-	250 µg/ml	BD Transduction
	1				Laboratories,
					Franklin Lakes,
					NJ.
Adaptin-β	Human β -adaptin	Mouse	IgG1	250 µg/ml	BD Transduction
					Laboratories
EEA-1	Amino terminal	Goat	-	200 µg/ml	Santa Cruz
	of human EEA-1				Biotechnology
					Inc., Santa Cruz,
					CA.
LAMP-1	Amino terminal	Goat	-	200 µg/ml	Santa Cruz
	of human LAMP-	, u	•		Biotechnology Inc
	1				
Clathrin HC	Carboxy terminal	Rabbit	-	200 µg/ml	Santa Cruz
	of human clathrin				Biotechnology Inc
	heavy chain				
Ferritin	Human ferritin L-	Rabbit	-	1.3 mg/ml	DAKO,
	chain				Carpinteria, CA.
Anti-	Transferrin	Rabbit	-	-	Gift from Dr. I.
transferrin	receptor				Trowbridge, The
receptor					Salk Institute, San
	· · ·				Diego, CA. [•]

Table IIIa: Primary antibodies used in the experiments presented in this thesis

[•] The rabbit anti-transferrin antiserum is used for western blotting

Antibody	Concentration	Dilution	Source
		used	
Alexa 488 nm	2 mg/ml	1:1000	Molecular Probes Inc.,
conjugated Goat anti-			Eugene, OR
mouse IgG		· · ·	
Alexa 568 nm	2 mg/ml	1:500	Molecular Probes Inc
conjugated Goat anti-			
rabbit IgG			
Alexa 568 nm	2 mg/ml	1:500	Molecular Probes Inc
conjugated Rabbit anti-			
mouse IgG			
Alexa 568 nm	2 mg/ml	1:500	Molecular Probes Inc
conjugated Rabbit anti-			
goat IgG			
Alexa 568 nm	2 mg/ml	1:500	Molecular Probes Inc
conjugated Goat anti-			
mouse			
Cy5 conjugated Rabbit	1 mg/ml	1:500	Jackson Laboratories,
anti-mouse IgG			West Grove, PA.

Table IIIb: Secondary antibodies used in the experiments presented in this thesis

II. Iron-binding and competition experiments

In preparation for the iron-binding and metal competition experiments, the cells were cultured in a 35 mm x 10 mm tissue culture dish (Nunc, Rochester, NY). Each dish was seeded with 3 x 10^5 cells that would grow to 100% confluency in 5 days. On the day of the experiment, each tissue culture dish was visually checked under the microscope for 100% confluency before commencing the experiment. The cells from two tissue culture dishes were counted separately by trypan blue exclusion method using a hemocytometer and averaged. The number of cells routinely used in each sample average between 1.4- 1.6×10^6 cells.

The method used to study the binding of radioactive iron to p97 is a modification of the binding assay published by Richardson and Baker (Richardson and Baker, 1991a; Richardson and Baker, 1991b). Briefly cells were washed 3 times with 1ml of Minimum essential media (MEM) (Invitrogen Life Technologies Inc., Burlington, Ont) supplemented with 1X MEM non-essential amino acids (NEAA)(Invitrogen Life Technologies Inc., Burlington, Ont) for 30 minutes at 37°C in a 5% CO₂ humidified environment. This was to ensure that excess iron and transferrin from the growth media were removed. A stock solution of 10 mM of ⁵⁵Fe-citrate was prepared by mixing ⁵⁵FeCl₃ in 0.5M HCl (Perkin Elmer Life Sciences Inc., Boston, MA) in citric acid at a 1:100 molar ratio to prevent hydrolytic precipitation of the iron complexes, which occur at physiological pH of the incubation media. Varying concentrations of ⁵⁵Fe-citrate were prepared by serial dilution in MEM supplemented with 20 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, 1.25 µg/ml bovine serum albumin (BSA) (Fisher Scientific Limited, Nepean, ON) and 1X MEM NEAA. P97⁺TRVb and p97 TRVb cell

lines were incubated with 1ml of the ⁵⁵Fe-citrate solution at 4°C. After 4 hours of incubation, the cells were washed 4 times with 1ml Hanks Balanced Salt Solution (Invitrogen Life Technologies Inc.) at 4°C. Phosphate buffered saline (PBS) (1ml) containing 0.25% (w/v) trypsin (Invitrogen Life Technologies Inc.) 1 mM EDTA (Sigma-Aldrich Canada Ltd) was added to the cells and incubated for 30 minutes at 37°C. The cell suspension was transferred into a scintillation vial and the tissue culture dish was rinsed with 0.5 ml of PBS and added to the scintillation vial. Beckman ready safe scintillation fluid (5ml) was added to the scintillation vial and the amount of radioactive decay was measured in the Beckman LS6000IS Liquid Scintillation counter (Beckman Coulter Inc., Fullerton, CA).

The radioactivity associated with each of the cell lines was normalized with the number of cells. Amount of radioactivity bound to GPI-anchored p97 was calculated by subtracting the amount of radioactivity associated with TRVb from that of p97⁺TRVb. Each sample was done in triplicate and the average plotted and standard deviation of the three samples were calculated and plotted as the error bar of the y-axis in Figure 5.

For competition experiment, a stock solution of 10 mM of metal citrate solution was prepared by mixing aluminum chloride, copper chloride and zinc chloride (all from Sigma-Aldrich Canada Ltd) with citric acid in a 1:100 molar ratio. The cells were then incubated for 4 hours at 37°C with 1µM of cold Fe-citrate, Al-citrate, Cu-citrate or Zncitrate in the presence of increasing concentrations of ⁵⁵Fe-citrate. At the end of the incubation, the cells were transferred to a 4°C chamber and washed four times with 1 ml of ice cold Hanks Balance Salt Solution. The cells were trypsinized and the radioactivity

associated with the cells is counted in the Beckman LS6000IS Liquid Scintillation counter.

III. Dialysis of soluble p97 and iron loading protocols

Soluble p97, graciously provided by Synapse Technologies Inc, Vancouver, BC, was partially saturated with iron. In order to load soluble p97 with other metals, iron contained in soluble p97 must be removed. The removal of iron is achieved by the dialysis protocol from Aisen et al (Aisen and Leibman, 1968; Aisen and Leibman, 1972). Briefly, 10 ml of approximately 1 mg/ml of soluble p97 was transferred into the Pierce 10,000 MW dialysis cassette and dialyzed in 0.1 M sodium citrate pH 6.0 in 4°C with constant stirring for an hour before the buffer was changed two more times. The protein was transferred to a new cassette and dialyzed for four hours in 0.1 M sodium perchlorate, 25 mM HEPES pH 7.4 at 4°C, with one change of buffer each hour. The cassette was then transferred to 25 mM HEPES, pH 7.4 dialysis buffer for three hours with one change of buffer each hour. The protein was concentrated in a Centricon-YM10 (Millipore, Bedford, MA) and the concentration of the protein was measured in a spectrophotometer at 280 nm. The concentration of apo-p97 was calculated based on the extinction coefficient of 1.214 (1% p97 solution) at 280 nm (Baker et al., 1992).

In order to ensure that the soluble p97 is in the apo-form, the dialyzed sample was electrophoresed in 6M urea polyacrylamide gel before metal loading experiment. Metal nitrilotriacetate (NTA) was prepared by mixing metal chloride with nitrilotriacetic acid-disodium salt (Sigma Chemical Co.) at ratio of 1:100. 1 nmoles of metal-NTA mixture was added to 0.1 nmoles of apo-p97. The mixture was neutralized with the addition of

sodium bicarbonate stock solution (10 mM) to pH 7.4. The mixture was allowed to incubate at room temperature for at least one hour before loading onto a 6M urea polyacrylamide gel.

IV. Urea polyacrylamide gel electrophoresis

The method of urea gel electrophoresis has been shown to resolve the four different species expected from partially iron-saturated transferrin. This method was first described by Makey and Seal (Makey and Seal, 1976). Briefly, a 6 % (w/v) polyacrylamide running gel was cast in a buffer of 0.089 M Tris-Cl, 0.089 M borate (pH 8.4) containing 6 M urea. The stacking gel was a 5% (w/v) polyacrylamide gel (Biorad) cast in 0.07 M Tris-Cl (pH 6.8) and 12% (w/v) sucrose. The running buffer used was 0.09 M Tris, 0.09M Borate and 0.0016 M EDTA pH 8.4. A stock solution of 10 M urea (Sigma Chemical Co) deionized in the AG 501-X8 Mixed Bed Resin (Biorad, Hercules, CA) and stored cold until used. One nanomole of the metal loaded p97 was prepared in 6X sample buffer (10% (w/v) sucrose (Fisher Scientific Limited), 0.002% (w/v) bromophenol blue (Fisher Scientific Limited), 0.09 M Tris (Fisher Scientific Limited), 0.09 M Borate (Fisher Scientific Limited) and 0.016 M EDTA, pH 8.4) and loaded onto the gel. A gradient of 3.5 V/cm was employed for electrophoresis at 4°C for 16-17 hours. The gel was fixed in 30% (v/v) methanol (Fisher Scientific Limited) and 10% (v/v) acetic acid (Fisher Scientific Limited) before staining with Coomassie Blue. The relative amount of each protein band is quantified by using densitometry tool provided in the Chemilmager 4000 Low light Imaging System (Alpha Innotech Corporation, San Leandro, CA).

V. UV-Visible spectroscopy study of iron binding to soluble p97

Soluble apo-p97 was dialyzed into 25 mM Tris-Cl, 10 mM NaHCO₃ and 100 mM NaCl pH 7.5. In order to determine the absorbance wavelength of iron saturated p97, 1 ml of soluble apo-p97 at 2 mg/ml, (equivalent to 20.53 nmoles of apo-p97), was transferred into the spectrophotometer cuvette. Iron nitrilotriacetate (Fe-NTA) complex was titrated into the protein solution at 4 nmole increments. After each addition of Fe-NTA, the sample was subjected to a scan in Cary 50 UV-Visible spectrophotometer (Varian Inc., San Diego, CA) from 380 nm to 600 nm in order to detect spectral changes in the absorbance spectrum.

To obtain the absorbance profile of p97 binding to iron, 4 nmole increments of iron were titrated into the protein solution. The absorbance at 475 nm was determined and plotted against the amount of iron added As a control, 1ml of 4 mg/ml apo-transferrin was titrated with 4 nmoles increment of FeNTA. The absorbance at 465 nm was determined and plotted against the amount of iron added.

VI. Differential scanning calorimetry

The differential scanning calorimetry (DSC) studies were performed on a MicroCal VP-DSC (MicroCal Inc., Northampton, MA). All experiments were carried out in 500 mM HEPES, 25 mM NaHCO₃, pH 7.5 at a scan rate of 60 °C /hr. The sample preparation protocol was obtained from Lin *et al* (Lin et al., 1993). Samples were degassed for 7 minutes with gentle stirring under vacuum prior to loading into the calorimeter. Protein concentrations were determined spectrophotometrically (280 nm)

using an extinction coefficient of 115,004 $M^{-1}cm^{-1}$. Samples containing iron were prepared by mixing appropriate amounts of protein, buffer and Fe-NTA (iron nitrilotriacetate) stock solution. The Fe-NTA stock solution was prepared by mixing 1 volume of 0.3 M FeCl₃ (in water) with 2 volumes of 0.3 M NTA, disodium salt (in water) and diluting to the appropriate concentration with buffer.

VII. Immunoprecipitation of ferritin.

TRVb, p97⁺TRVb and TfR⁺TRVb cell lines were separately were grown on 60 mm x15 mm tissue culture dish to 100% confluency. The cells were washed twice, 30 minutes each time in Iscove's modified Dulbecco media (IMDM) (Invitrogen Life Technologies Inc.) at 37°C to remove endogenous iron and serum transferrin. They were treated either with or without 300 mU of phosphoinositol-phospholipase C (PI-PLC) (Roche, Basel, Switzerland) in IMDM for 30 minutes at 37°C. PI-PLC is an enzyme that removes GPI-anchor from a subset of GPI-anchored protein, which includes GPIanchored p97. The cells were then incubated with either 1.5 ml of 1 μ M of ⁵⁵Fe nitrilotriacetate (NTA) or 2 mg/ml ⁵⁵FeNTA loaded human transferrin (Sigma-Aldrich Canada Ltd) for 90 minutes at 37°C in the presence of the PI-PLC in a 5% CO₂ atmosphere. The cells were washed twice with ice cold PBS before lysing them in 1 ml of 1% (v/v) Nonidet-P40 (NP-40)(ICN Pharmaceuticals Inc, Costa Mesa, CA) in lysis buffer (20 mM Tris-Cl pH 7.4, 150 mM NaCl, 2 mM EDTA and 1 tablet of protease inhibitor cocktail (Roche) for 30 minutes on ice. The lysate was collected and centrifuged in Biofuge B (VWR Canlab Mississauga Ont) at 11,000 rpm at 4°C for 10 minutes. The supernatant was collected and pre-cleared at 4° C with 3 μ l of normal rabbit

serum for 1 hour followed by another hour with 30 μ l of protein G sepharose (50% (v/v) slurry) (Amersham-Pharmacia Biotech, Piscataway, NJ). The lysate (150 μ l) was aliquoted into eppendorf tubes and immunoprecipitated with antibodies against human p97 (L235), human transferrin receptor (OKT9) and human ferritin. Protein G sepharose (30 μ l) was used to precipitate the immuno-complexes. The protein G-immuno-complexes were washed twice in buffer B (0.2% (w/v) NP-40, 10 mM Tris-Cl pH 7.5, 150 mM NaCl, 2 mM EDTA), once in buffer C (0.2% (w/v) NP-40, 10 mM Tris-Cl pH 7.5, 500 mM NaCl, 2 mM EDTA) and once in buffer D (10 mM Tris-Cl pH 7.5) before resuspension in 1 ml of PBS. The PBS suspensions were transferred to scintillation vials and 5 ml of ReadySafe scintillation counting fluid (Beckman Coulter Inc., Fullerton, CA) were added. The radioactive counts associated with the immunoprecipitate were using the Beckman LS6000IS Liquid Scintillation counter.

VIII. Endocytosis inhibition experiment

 $p97^{+}TRVb$, TfR⁺TRVb and untransfected TRVb cell lines were grown on 35 mm x 10 mm tissue culture plates until 100% confluent. TRVb is a CHO cell line, which has a defective endogenous hamster transferrin receptor. The cells were pre-incubated with the 250 µg/ml nystatin (Sigma-Aldrich Canada Ltd) and 25 µg/ml filipin (Sigma-Aldrich Canada Ltd) both prepared in di-methyl-sulfoxide (DMSO) (Sigma-Aldrich Canada Ltd) for 30 minutes before 1µM of ⁵⁵Fe-citrate or 1 mg/ml of ⁵⁵Fe-transferrin was added to the cells and incubated for 2 hours at 37°C. As a control, the cells were also treated with an equal amount of DMSO as present in the inhibitors. The cells were washed with 1 ml of PBS and harvested with a cell scraper in the presence of 1 ml Versene (2 mM EDTA in

PBS) and transferred to scintillation vials. The tissue culture plate was rinsed with 0.5 ml of PBS and the rinse was added into the scintillation vial. Five milliliters of scintillation counting fluid were added and the radioactivity count associated with the immunoprecipitate was determined in Beckman LS6000IS Liquid Scintillation counter.

The experiments were performed in triplicate. The radioactive counts associated with each samples were averaged. The radioactivity associated with the TRVb samples was subtracted from the $p97^+$ TRVb and TfR⁺TRVb samples.

IX. Immunofluorescence staining and confocal laser scanning microscopy

SK-MEL 28 cells were grown on sterile No.1 18 mm square coverslips (Fisher Scientific Inc.) to 70% confluency. The coverslip was washed twice in PBS before blocking it with 2% (w/v) BSA/ PBS for 1 hour at 4°C. The coverslip was stained with 200 μ l of anti-p97 (L235) or anti-transferrin receptor (OKT9) antibodies at 1: 500 dilution in 2% (w/v) BSA/ PBS for 30 minutes on ice. Excess antibodies were washed off five times for 10 minutes with 2% (w/v) BSA/ PBS before transferring the coverslips to pre-warmed 2 % (w/v) BSA/ PBS and incubating them at 37°C for 0, 10, 20 and 30 minutes to allow endocytosis to occur. After the incubation period, the cells were fixed in 2 % (w/v) paraformaldehyde (BDH Laboratory Supplies, Dorset, England) in PBS for 20 minutes at room temperature. A second antibody was used to stain for the various vesicles. Rabbit anti-caveolin antibody (250 μ g/ml) was diluted at 1: 50, goat anticlathrin antibody (200 μ g/ml) was used at 1: 40. All antibodies were diluted in 2% (w/v)

BSA/ PBS. The cells were stained for 30 minutes at room temperature before five washes for 10 minutes with 0.1% (w/v) saponin, 2% (w/v) BSA/ PBS. Alexa 488, Alexa 568 or Cy5 conjugated secondary antibodies were used to visualize the staining pattern (Table IIIb). The secondary antibodies are diluted in 2% (w/v) BSA/ PBS. Two hundred microliters of diluted secondary antibodies were used to stain the cells at room temperature for 20 minutes, followed by five washes for 10 minutes with 0.1% (w/v) saponin, 2% (w/v) BSA/ PBS. The coverslip was mounted with 5 μ l of Slow Fade (Molecular Probes, Eugene, OR) before sealing it onto a glass slide with nail polish.

For triple antibody labeling, SK-MEL 28 cells grown on coverslips were washed three times 15 minutes in IMDM at 37°C in order to remove serum transferrin. The coverslips were blocked with 2% (w/v) BSA/ PBS for 1 hour on ice and stained with 200 μ l of 5 μ g/ml Alexa 488 conjugated transferrin and 200 μ l of anti-p97 antibody (L235) diluted at 1:500 for 30 minutes on ice. Excess antibody and Alexa 488 conjugated transferrin were washed off with PBS and the cells were allowed to undergo endocytosis for 0, 10, 20, 30 minutes in 2% (w/v) BSA/ PBS pre-warmed to 37°C. The cells were fixed in 2% (w/v) paraformaldehyde for 15 minutes at room temperature followed by a 15-minute permeabilization step in 0.1% (w/v) saponin at room temperature. Goat anti human EEA-1 antibody was used to stain the endosomal marker for 30 minutes followed by five washes in PBS for a total of 10 minutes. Alexa 568 and Cy-5 conjugated secondary antibodies were used to visualize the staining pattern (Table IIIb). The secondary antibodies are diluted in 2% (w/v) BSA/ PBS. Excess secondary antibody was removed and the coverslip was mounted onto a glass slide with Slow-Fade. The BioRad Radiance Plus confocal laser scanning microscope was used to capture all the fluorescent images and NIH imaging system was used to analyze the images.

X. Cryoimmunoelectron microscopy

Two 140 mm x 20 mm plates of SK-MEL 28 cells were grown to 90% confluency and washed twice with 10 ml of pre-warmed (37°C) PBS before fixing in 2% (w/v) paraformaldehyde at 37°C for 30 minutes. The fixed cells were washed twice with PBS and scraped off gently in 1 ml of PBS with a rubber policeman and centrifuged at maximum speed in the Biofuge B centrifuge. The pellet was infused overnight with 25% (w/v) polypyrollidone (Sigma-Aldrich Canada Ltd) in 2.3 M sucrose at 4°C. The next day, the pellet was trimmed with a razor blade and a small pyramid shaped block was mounted on the aluminum specimen pin (EM Sciences, Hawthorne, NY) with a drop of O.C.T compound (Tissue-Tek, Elkhart, IN). The specimen pin was quickly frozen in liquid nitrogen. Eighty-nanometer sections were cut with a glass knife at -110 °C and sections were picked up with 2.3M sucrose onto a formvar-coated 200 meshed nickel grid (EM Sciences). Before blocking the grid with 0.5% (v/v) fish skin gelatin (Sigma-Aldrich Canada Ltd) in PBS for 10 minutes, the grid was washed in PBS four times within 10 minutes. Rabbit anti-caveolin antibodies (50 μ l) at 1:100 dilution, L235 (50 μ l) at 1:100 dilution, goat anti-clathrin antibody (50 µl) at 1: 100 dilution and OKT-9 (50 µl) at 1:100 dilution were used to stain the grids. All the antibodies were diluted in 0.5%(v/v) fish skin gelatin in PBS. The grids were stained with primary antibody for 20 minutes at room temperature and washed four times in PBS before staining with secondary antibody conjugated to gold. Goat anti-rabbit conjugated to 10 nm gold (50 μ l), goat anti-mouse conjugated to 5 nm gold (50 μ l) and donkey anti-goat conjugated to 10 nm gold (50 μ l) antibodies were used at 1:50 dilution in 0.5% (v/v) fish skin gelatin. After staining for 20 minutes in secondary antibody, the grids were rinsed 4 times in PBS and washed 4 times for 15 minutes in PBS followed by 6 rinses in distilled water for 20 minutes. The grid was then sealed and counter-stained with 2% (w/v) aqueous uranyl acetate (EM Sciences Inc.) in methyl cellulose (Sigma-Aldrich Canada Ltd). Excess liquid was blotted off with Whatman filter paper no.1 (VWR Canlab) and the grid was air-dried before examining it under the Zeiss EM 10C transmission electron microscope at 80 kV (Zeiss, Esslingen, Germany).

XI. Sucrose gradient sub-cellular fractionation and western blotting

Three confluent 140 mm x 20 mm plates of SK MEL-28 cells with a total of 3 $\times 10^7$ cells were washed two times with cold PBS. Cells were scraped with a rubber policeman in 5 ml of PBS and centrifuged in Beckman GPR centrifuge (Beckman Coulter Inc.) for 5 minutes at 4°C at 1,200 rpm. The pellet was lysed on ice in 1 ml 1% (v/v) Triton X-100 in 140 mM KCl, 10 mM Tris-Cl, pH 7.5 for 30 minutes. One milliliter of 80% (w/v) sucrose in 140 mM KCl, 10 mM Tris-Cl, pH 7.5 was added to the lysate to make a 40% (w/v) sucrose lysate mixture. The lysate mixture was transferred into a 10 ml polypropylene tube and overlaid with 6 ml of cold 30% (w/v) sucrose in 140 mM KCl, 10 mM Tris-Cl, pH 7.5, followed by 3.5 ml of 5% (w/v) sucrose in 140 mM KCl, 10 mM Tris-Cl, pH 7.5. This step gradient was then centrifuged in Sorvall RC80 ultracentrifuge with a Sorvall TH-641 rotor (DuPont Canada Inc., Mississauga ON), at 39,000 rpm for 16 hours at 4°C. A white flocculent band was observed at the interface

between the 5% (w/v) and 30% (w/v) sucrose gradient. Eight fractions of 1.5 ml were collected and the pellet was resuspended in lysis buffer.

Each fraction (20 µl) was mixed with 10 µl of 3X reducing sample buffer (250 mM Tris-Cl, 3% (v/v) glycerol, 0.16% (w/v) SDS, 0.002% (w/v) bromophenol blue and 1% (w/v) dithiothreitol (DTT) (Roche)). The mixture was heated at 95°C for 5 minutes and allowed to cool down to room temperature before 5 μ l of 0.5 M iodoacetamide (Sigma-Aldrich Canada Ltd) was added to it. The sample was then loaded onto a 10% (w/v) SDS-PAGE and electrophoresed at 150 V for 1 hour. The gel was transferred to Immobilon membranes (Biorad) by electroblotting. The membrane was blocked with 5% skim milk, 0.1% (v/v) Tween-20 (Biorad) in PBS for 2 hours and detected using antibodies against caveolin (1: 100 dilution), p97 (1: 100 dilution), transferrin receptor (1: 100 dilution) and β-adaptin (1: 100 dilution) in 5% (w/v) skim milk, 0.1% (v/v) Tween-20 in PBS overnight at 4°C. After 4 washes of 30 minutes each in 5% (v/v) skim milk 0.1% (v/v) Tween-20 in PBS, peroxidase conjugated goat anti-mouse and peroxidase conjugated goat anti-rabbit antibodies (1:10,000 dilution) in 5% (w/v) skim milk, 0.1% (v/v) Tween-20 (Biorad) in PBS were used to detect the primary antibodies. The chemiluminescence ECL Western blotting detection system (Amersham Pharmacia Biotech) was used to detected the chemiluminescence.

In order to determine whether iron transported by GPI-anchored p97 fractionates into the caveolae fraction. SK-MEL 28 cells were labeled with 1.5 ml of 1 μ M of iron-55 nitrilotriacetate (⁵⁵Fe-NTA) for 15 minutes at 37°C in a 5% CO₂ atmosphere before being subjected to sub-cellular fractionation. P97 was immunoprecipitated from each fraction. The radioactivity associated with the immunoprecipitate complex was determined using a

Beckman LS6000IS Liquid Scintillation counter. The amount of radioactivity represents radioactivity associated with GPI-anchored p97 after subtraction of radioactivity associated with normal rabbit serum (NRS).

XII. Transfection of Nramp2 into SK-MEL 28 cells

SK-MEL 28 cells were grown to 50% confluency on sterilized No.1 18mm square glass coverslips. 2 µg of pEGFP-hNramp2 (human Nramp2 subcloned into Enhanced green fluorescence protein vector from Clontech (Clontech, Palo Alto, CA) (gift from Dr. F. Kishi, Center for Gene Research, Yamaguchi University, Ube, Yamaguchi 755-8505 Japan) was transfected into SK-MEL 28 cells using Fugene 6 (Roche) following the protocol supplied by the manufacturer. The cells were stained for GPI-anchored p97 or transferrin receptor 24-hour after transfection following the immunofluorescence protocol. EGFP was observed directly.

Chapter 3: Metal binding properties of p97

I. Other metals can compete with iron for p97

A. <u>Rationale</u>

The best-characterized pathway of iron uptake is the transferrin receptor mediated endocytosis of transferrin. However, the *in situ* distributions of iron and transferrin in the brain are not in the identical regions (Dwork et al., 1988; Hill and Switzer, 1984). Therefore, there is debate on whether transferrin mediates the transport of metals into the brain. Metals such as iron, copper, aluminum and zinc are known to play roles in the pathology of several neurodegenerative disorders such as Alzheimer s and Parkinson s diseases (Bush, 2000; Bush et al., 1994; Perl and Pendlebury, 1986; Sayre et al., 2000). It is yet unknown how these metals are transported into the brain, however, one possible candidate for metal transport into the brain is p97. The level of soluble p97 in the serum and cerebrospinal fluid is elevated in Alzheimer s disease patients (Kennard et al., 1996; Kim et al., 2001). In addition, soluble p97 is also able to cross the blood-brain barrier into the brain more efficiently than transferrin (Moroo et al., 2001). One hypothesis suggests p97 is able to mediate the transport of these metals into the brain.

Before establishing p97 as a candidate for metal transport into the brain, it was crucial to determine whether these metals could bind to p97. It is known that metals such as aluminum, copper, zinc and others are able to bind to the same iron-binding site as iron in transferrin. (Congiu-Castellano et al., 1997; Harrington et al., 1987; Harris, 1983; Harris and Stenback, 1988; Hutchens and Yip, 1991; Roskams and Connor, 1990). Therefore, due to the high protein sequence homology between transferrin and p97, p97 may also bind these other metals. In addition, there is a consensus zinc binding sequence

in between the N-lobe and the C-lobe of p97 (Garratt and Jhoti, 1992). It is not known whether zinc could bind to p97 at this motif or whether zinc can bind to the iron-binding site. Furthermore, the close proximity of the zinc-binding site to the two iron-binding sites can affect the binding kinetics of iron to GPI-anchored p97. A recent review suggested that p97 might be a zinc-dependent metalloprotease (Sekyere and Richardson, 2000). However, the protease activity has not been demonstrated.

Two sets of experiments to examine whether copper, aluminum and zinc could bind to p97 were carried out. The competition experiment examined whether these metals will bind to the GPI-anchored p97 and the urea polyacrylamide gel electrophoresis analyzed whether these metals could bind to soluble p97.

B. <u>Result I: Aluminum, copper and zinc can compete with iron for GPI-anchored</u> p97

Radioactive copper, zinc and aluminum emit very high-energy β -particles and are dangerous to work with. An indirect approach to examine whether these metals bind to GPI-anchored p97 is to determine whether they can compete for the iron-binding site of GPI-anchored p97 with radioactive iron. Transferrin receptor deficient Chinese hamster ovary cells transfected with or without p97 (p97⁺ TRVb and p97⁻TRVb) were incubated with varying concentrations of ⁵⁵Fe-citrate in the presence or absence of 1µM of cold metal citrate complexes at 37°C for four hours. After the incubation, the cells were washed and the radioactivity associated with p97⁻ and p97⁺ transfected TRVb cell lines were determined and the difference in radioactivity between the supernatants of both cell

lines was plotted (Fig. 5). Therefore, the amount of ⁵⁵Fe-citrate plotted represents ⁵⁵Fe-citrate bound to GPI-anchored p97.

Figure 5 shows the amount of ⁵⁵Fe-citrate bound to GPI-anchored p97 at 37°C in the absence of cold Fe-citrate is approximately 4.2 picomoles per million cells when the cells were incubated with 2 μ M or more ⁵⁵Fe-citrate. However, the addition of 1 μ M of cold iron citrate, copper citrate or aluminum citrate reduced the amount of ⁵⁵Fe-citrate bound to GPI-anchored p97 to 2.2, 2.5 and 2.4 picomoles per million cells, respectively after 4 hours (Fig 5A, 5B, 5C). This indicates that cold iron, copper and aluminum compete with ⁵⁵Fe-citrate for the iron-binding site in GPI-anchored p97.

Interestingly, the binding of ⁵⁵Fe-citrate to GPI-anchored p97 in the presence of 1μ M zinc-citrate was dramatically different from that of copper citrate and aluminum citrate. In this case, the total amount of ⁵⁵Fe-citrate bound to GPI-anchored p97 was not affected by the presence of 1μ M zinc citrate. GPI-anchored p97 was able to bind increasing amount of ⁵⁵Fe-citrate per million cells in the presence of 1μ M of zinc-citrate, however, higher concentrations of ⁵⁵Fe-citrate were required for the same number of cells to bind the same amount of ⁵⁵Fe-citrate (Fig. 5D). This indicates that although the presence of zinc does not affect the overall amount of ⁵⁵Fe-citrate bound to GPI-anchored p97.

These competition experiments show that copper and aluminum competed for the iron-binding site in GPI-anchored p97, whereas zinc did not affect the total amount of iron bound to p97.

Figure 5: The effects of cold metal citrate competitors on the amount of ⁵⁵Fe-citrate bound to GPI-anchored p97. (A) Cold Fe-citrate (B) cold Al-citrate and (C) cold Cucitrate reduces the amount of ⁵⁵Fe-citrate bound to GPI-anchored p97. (D) Cold Zncitrate reduces the amount of ⁵⁵Fe-citrate binding to GPI-anchored p97 at low ⁵⁵Fe-citrate concentrations but does not reduce the amount of ⁵⁵Fe-citrate bound to GPI-anchored p97 at high ⁵⁵Fe-citrate concentrations. The error bars represent the standard deviations of three samples.



C. <u>Results II: Soluble p97 can bind iron, copper and zinc</u>

Several investigators have shown that partially iron-saturated human serum transferrin can be resolved into four discrete bands corresponding to: apo-transferrin, C-lobe saturated transferrin, N-lobe saturated transferrin and holo-transferrin, in the order of increasing mobility when the sample is subjected to 6 M urea polyacrylamide gel electrophoresis (Funk et al., 1990; Makey and Seal, 1976; Penhallow et al., 1991). The presence of 6M urea causes the lobes that do not contain iron to unfold causing different mobility during electrophoresis (Evans and Williams, 1978). The molecular basis for the electrophoretic separation appears to be related to two observable facts: (1) the increase in protein stability as iron is bound to transferrin and (2) the asymmetrical distribution of amino acids between the two lobes of the transferrin molecules (Evans and Williams, 1978; Makey and Seal, 1976). Since p97 is structurally similar to transferrin, this technique was applied to examine whether soluble p97 loaded with different metals exhibits an altered mobility on the urea-polyacrylamide gel.

As a control, partially and fully saturated human serum transferrins were subjected to 6 M urea polyacrylamide gel electrophoresis. Lane 1 in Figure 6A shows that partially saturated human transferrin can be separated by mobility into four distinct bands as mentioned in previous observations by Makey and Seal. Fully saturated human transferrin exists as a single band (Fig. 6A lane 2).

Apo-soluble p97 was incubated with excess amount of iron nitrilotriacetate (Fe-NTA), aluminum nitrilotriacetate (Al-NTA), copper nitrilotriacetate (Cu-NTA), or zinc nitrilotriacetate (Zn-NTA). The protein was electrophoresed onto a 6% urea-

Figure 6: Urea polyacrylamide gels of human serum transferrin and human soluble p97. (A) Partially iron-saturated (lane 1) and fully iron-saturated human serum transferrin (lane 2) and (B) dialyzed p97 (lane 1), p97 loaded with either aluminum nitrilotriacetate (Al-NTA) (lane 2), copper nitrilotriacetate (Cu-NTA) (lane 3), iron nitrilotriacetate (Fe-NTA) (lane 4), or zinc nitrilotriacetate (Zn-NTA) (lane 5) and undialyzed p97 (lane 6). This is a representative urea polyacrylamide gel from two separate experiments.



(B)

(A)



polyacrylamide gel and the gel was fixed with methanol and stained with Coomassie blue. The relative amount of each band of protein in each lane was quantitated using 1D-Multi (line densitometry) (Fig. 6B). Table IV shows relative amount of protein in each lane corresponding to the protein bands in Figure 6B. Lane 6 represents the protein before dialysis presented as two different forms of soluble p97. The upper band represents approximately 60% of the protein and the lower band represents 40% of the protein. After extensive dialysis, only the upper band of the protein exists, as shown in lane 1. Therefore, the upper band seen in lane 6 is the apo-form of soluble p97 whereas the lower band is soluble p97 bound to iron. Lane 4 shows that when the soluble apo-p97 is saturated with iron, a large fraction of the protein (85%) shifted to the lower band and a small fraction of the protein (15%) shifted to an even lower band. The identity of these bands will be discussed later.

It is shown in lane 2 that when apo-p97 is loaded with Al-NTA, there is very minimal shift of protein to the lower band. It is shown in lane 3 that soluble apo-p97 binding to Cu-NTA. A slightly stronger binding is observed for Cu-NTA compared with Al-NTA, approximately 29.8 % of the protein shifts to the lower band while 70.2 % of the protein remains in the apo-form. The protein distribution in lane 5 shows that p97 binds to moderate amounts of Zn-NTA. Fifty percent of the protein shifts to the lower band whilst the other 50% remains in the apo-form.

In summary, although metals such as copper and zinc show a degree of binding to soluble p97, the binding of iron to the protein is preferred. Aluminum did not appear to bind to soluble p97 whereas zinc is able to bind to soluble p97 as observed in the urea-polyacrylamide gel.

Table IV:	Relative amount	of metal l	loaded s	soluble p	97 in	the urea	polyacryla	mide gel
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	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6
Metal	-	Al	Cu	Fe	Zn	-
Band 1	100%	100%	70.2%	-	42.7%	60.2%
Band 2	0%	0%	29.8%	85.1%	57.3%	39.8%
Band 3	-	-	-	14.9%	-	-

D. <u>Discussion</u>

One goal of this thesis is to understand the metal binding properties of p97. The GPI-anchored form has been shown to bind iron and internalize it into the cell (Kennard et al., 1995). The function of the soluble form, however, is not yet established. It is known that the level of soluble p97 is higher in the serum of Alzheimer s disease patients compared to age matched control group (Kennard et al., 1996). Therefore, questions arise as to the role of soluble p97 in Alzheimer's disease. There is increasing evidence to show that metals are important factors in the pathology of several neurological disorders. Iron, zinc, copper and aluminum have all been implicated in the disease progression of Alzheimer s disease (Bush, 2000; Bush et al., 1994; Lynch et al., 2000; Miura et al., 2000; Moir et al., 1999). It is not known how these metals are transported from the serum across the blood-brain barrier into the brain. Initially, it was thought that transferrin could mediate the transport of these metals into the brain since it has been shown that transferrin can also bind metals other than iron (Congiu-Castellano et al., 1997; Harrington et al., 1987; Harris, 1983; Harris and Stenback, 1988; Hutchens and Yip, 1991; Roskams and Connor, 1990). However, based on accumulated evidence, it is highly unlikely that transferrin and transferrin receptor are the only factor that governs the transport of metals across the blood-brain barrier. This prospect is supported by the following observations: (1) There is little overlap between the distribution of transferrin receptor and iron in the normal, Alzheimer's disease and Parkinson's disease brains (Dwork et al., 1988; Hill and Switzer, 1984; Schipper, 1999). (2) The concentration of iron exceeds the iron-binding capacity of transferrin in the cerebral spinal fluid.

Therefore, there may be other candidates involved in the transport of iron (Beard et al., 1993). (3) Hpx mice have higher than normal iron uptake in the brain (Trenor et al., 2000). Therefore, an alternate route of iron uptake independent of transferrin must exist in these mice since non-transferrin bound iron seems to be able to cross the blood-brain barrier in these mice (Ueda et al., 1993). p97 is one such candidate.

The tissue distribution studies of p97 indicate that it is expressed by the human brain capillary endothelium (Rothenberger et al., 1996) and is produced in reactive microglial associated with senile plaque in Alzheimer s disease (Yamada et al., 1999). In addition, there is increasing evidence suggesting that metals such as iron, copper, zinc and aluminum play a role in precipitating the amyloid plaques in Alzheimer s disease (Bush, 2000; Bush et al., 1994). Therefore, before examining whether p97 can mediate transport of these metals into the brain *in vivo*, a series of *in vitro* competition assay between iron and other metals for GPI-anchored p97 and metal loading of soluble p97 were carried out in order to determine whether p97 can bind these metals *in vitro*.

The competition experiment show that copper and aluminum competed for the iron-binding site in GPI-anchored p97, whereas zinc did not affect the total amount of iron bound to p97. Urea polyacrylamide gel electrophoresis of soluble p97 loaded with different metals shows that although copper and zinc are able to bind to soluble p97, the binding of iron to the protein is preferred. Aluminum, however, did not appear to bind soluble p97.

a. <u>Copper, aluminum and zinc can compete with iron for binding to GPI-anchored</u> p97

The competition experiment between ⁵⁵Fe-citrate and cold metal citrate demonstrated that the presence of copper-citrate and aluminum-citrate decreased the total amount of ⁵⁵Fe-citrate internalized by GPI-anchored p97 (Fig. 5). This suggests that copper-citrate and aluminum-citrate can compete with ⁵⁵Fe-citrate for binding to GPI-anchored p97. Crystallographic studies of human copper-lactoferrin have shown that the metal binding site for copper is the same as that for iron (Smith et al., 1992). Since the iron-binding site in the N-lobe of human p97 is conserved, it is possible that iron and copper have the same binding site in human p97. Thus, copper is able to compete with iron for p97 and hence the decrease in ⁵⁵Fe citrate binding to GPI-anchored p97 in the presence of cold copper-citrate.

The interaction of aluminum with human serum transferrin and lactoferrin has been examined using X-ray absorption near edge structure (XANES) spectroscopy, circular dichroism, UV-visible, and fluorescence spectrometry (Congiu-Castellano et al., 1997; Tang et al., 1995). These studies illustrate that aluminum can bind to human serum transferrin and lactoferrin. Figure 5 shows that aluminum-citrate competes with ⁵⁵Fecitrate binding to p97 suggesting p97 is able to bind to aluminum.

The presence of zinc-citrate also affects the binding of ⁵⁵Fe-citrate to GPIanchored p97. Interestingly, the competition profile of zinc-citrate is different from the other three metal-citrate complexes. Higher concentrations of ⁵⁵Fe-citrate are needed for GPI-anchored p97 to bind the same amount of ⁵⁵Fe-citrate in the presence of zinc-citrate compared to that when zinc-citrate is absent. The total amount of ⁵⁵Fe-citrate bound to GPI-anchored p97 was not affected by the presence of 1 μ M of zinc-citrate (Fig. 5D). This indicates that zinc may have a different binding site in GPI-anchored p97 or that zinc may have a weak binding affinity for p97. An elegant molecular modeling study has identified a potential zinc-binding consensus sequence (HEXXH) present between the N-and the C- lobes of p97 (Garratt and Jhoti, 1992) (Fig. 2). Therefore, it is possible that zinc does not compete with iron for the binding sites directly but that zinc may affect the binding of iron to GPI-anchored p97 by causing some stereo-spatial distortion due to the close proximity of the zinc-binding site to the two iron-binding sites. The molecular modeling also suggests that the stereochemical orientation of the zinc-binding site is super-imposable with the zinc-binding site of thermolysin. This observation led to a proposal that p97 might have zinc dependent metalloprotease activity (Garratt and Jhoti, 1992). However, only the human orthologue of p97 contains this zinc binding consensus sequence for metalloprotease activity while p97s from other species have somehow lost this function. Experiments to determine whether p97 can function as a metalloprotease are in progress.

b. <u>The binding of iron, copper, aluminum and zinc to soluble p97</u>

The availability of soluble p97 presented an opportunity to use other methods to study whether metals other than iron, bind to p97. Several investigators have shown that partially saturated transferrin can be differentiated into four different forms by electrophoresis in a medium containing 6M urea and a Tris/ borate/ EDTA, pH 8.4 buffer (Evans and Williams, 1978; Makey and Seal, 1976). This is based on evidence from peptide mapping of transferrin that indicates transferrin is not symmetrical with respect to amino acid composition between the two lobes and the increase in structural stability as

the protein binds to iron. The four different forms are apo-transferrin, C-lobe saturated transferrin, N-lobe saturated transferrin and holo-transferrin and they show increased mobility on a 6 M urea polyacrylamide gel as depicted in Figure 6A and Figure 7 (Funk et al., 1990; Makey and Seal, 1976; Penhallow et al., 1991). A similar profile is expected if metals such as iron, copper, aluminum and zinc can bind p97. It is shown in lane 4 of Figure 6 shows that in the presence of iron, majority of the soluble p97 (85%) exists as slower migrating upper band. A smaller but significant fraction of the protein (15%) exists as a faster migrating lower band. There are three possible interpretations as to the identity of these two bands (Fig. 7). In comparison to the transferrin profile, one possibility is that the major band seen in Figure 6 lane 4 corresponds to the iron bound Clobe and the minor band corresponds to iron bound to the N-lobe (situation 1 in Fig. 7). However, this is unlikely since the iron-binding site in the N-lobe is conserved in both p97 and transferrins but the iron-binding site in the C-lobe is not. Therefore, the upper major band can be assumed to be the iron bound N-lobe and the lower minor band corresponds to the iron bound C-lobe (situation 2 in Fig. 7). There is no additional band of higher mobility than the minor band, indicating the absence of the fully saturated soluble p97. This data would suggest that the C-lobe of soluble p97 is able to bind to iron. Soluble apo-p97 was loaded with excess amount of Fe-NTA before electrophoresis on a 6 M urea-polyacrylamide gel. However, the fact that iron loaded N-lobe was present at a greater proportion than iron loaded C-lobe and there was no fully saturated soluble p97 suggests that the two lobes bind to iron with very different affinity. Perhaps the amino acid substitutions in the C-lobe have greatly affected its ability to bind iron. There is another possible interpretation for the presence of the two different bands (situation 3



in Fig. 7). Data from the urea polyacrylamide gel electrophoresis of the partially saturated transferrin suggested that the order of resolution of the different forms of transferrin is apo-transferrin, C-lobe loaded transferrin, N-lobe loaded transferrin and fully saturated transferrin in the order of increasing mobility. Therefore, an alternative explanation is that the upper major band is the N-lobe loaded with iron and the lower minor band is the fully saturated p97. Under this interpretation, the band corresponds to iron bound C-lobe alone is not detected. Nevertheless, both scenarios 2 and 3 suggest that the major band is the N-lobe can bind iron, albeit weakly.

Urea polyacrylamide gel electrophoresis also demonstrates that zinc and copper can bind to the soluble p97 whereas aluminum shows little or no binding to the protein (Fig. 6). The binding of Cu-NTA to soluble p97 as shown by the urea polyacrylamide gel (Fig. 6 lane 3) agrees well with the data from the competition experiment showing the ability of copper-citrate to compete with ⁵⁵Fe-citrate for GPI-anchored p97 (Fig. 5C). The competition data suggests that cold aluminum-citrate can interfere with the binding and uptake of ⁵⁵Fe-citrate by GPI-anchored p97 (Fig. 5). However, the urea polyacrylamide gel electrophoresis data suggests that soluble p97 does not bind aluminum. One explanation for this discrepancy is that there is a significant increase in structural distortion around the metal site when aluminum tries to bind to it. The structural study of aluminum and transferrin shows that the aluminum ion is hexa-coordinated in an octahedral-like symmetry in the iron coordination site and thus creates a contortion that results in unstable coordination between aluminum ion and transferrin (Congiu-Castellano et al., 1997). Therefore, in a competitive binding assay, the presence of cold aluminum-citrate is enough to interfere with the binding of ⁵⁵Fe-citrate to GPI-anchored

p97. However, in a urea gel assay, aluminum-citrate is probably not tightly bound to soluble p97 and therefore when the protein is electrophoresed in the urea gel, the aluminum ion probably detaches from the protein.

The competition data demonstrate that zinc-citrate does not affect the total amount of ⁵⁵Fe-citrate binding to GPI-anchored p97. One possible interpretation given is that zinc does not bind to the iron-binding site in p97. The urea polyacrylamide gel electrophoresis of Zn-NTA loaded soluble p97 shows that zinc is able to bind to soluble p97 and shifts approximately 50% of the apo-p97 to a higher mobility form of zinc bound soluble p97. There are two possible interpretations of this shift in mobility. It is possible that in the absence of competing iron atoms, zinc is able to bind to the iron bind site and therefore the lower band shown in Figure 6 lane 5 is zinc binding to N-lobe of p97. However, another interpretation is that Zn-NTA binds to a different site. The proposed zinc-binding site of p97 is located in between the N-and the C-lobes (Figs.1 and 2). Zinc could bind to the proposed site of soluble p97 and shift the mobility of the protein to the lower band.

Furthermore, the absence of the apo-form of p97 when the protein is loaded with iron compared to the presence of the apo-form of p97 when it is loaded with copper, aluminum or zinc, suggests that p97 binds preferentially to iron.

II. The iron-binding stoichiometry of soluble p97

A. <u>Rationale</u>

The members of the transferrin family share a conserved tertiary protein structure. The typical structure is a single chain glycoprotein, which is divided into two similar but

not quite identical lobes, the N-lobe and the C-lobe representing the N-terminal and Cterminal halves of the molecule. Each lobe, in turn, comprises two domains surrounding a deep hydrophilic cleft enclosing an iron-binding site. The iron-binding site is made up of four conserved amino acid residues: an aspartic acid, two tyrosines and one histidine. They provide the coordination sites for the iron atom. A threonine and an arginine provide a network of electrostatic and hydrogen bonds for the synergistic anion. Sequence alignments of p97 and other members of the transferrin family show that the amino acids involved in coordinating the iron atom of the N-lobe are totally conserved (Fig. 1). However, there are the four amino acid substitutions (D421S, D482S, T478 and T483P) in the iron and anion binding sites in the C-lobe of human p97. These substitutions have been proposed to affect the iron-binding ability of the C-lobe. A previous study by Baker et al showed that p97 bound iron at 1:1 ratio (Baker et al., 1992). However, the urea-polyacrylamide gel data in the previous section showed that there was a possibility that the C-lobe of p97 can bind iron (Fig. 6). In addition, X-ray crystallography of the N-lobe D60S human lactoferrin, which mirrors the D421S substitution in human p97, suggests that even when the aspartic acid is substituted, the recombinant human lactoferrin is still able to bind iron (Faber et al., 1996). Furthermore, the binding constant of iron for p97 has never been determined. Therefore, UV-visible spectroscopy was performed on the soluble form of p97 to determine the stoichiometry of iron to p97.
B. <u>Results I: Determination of the peak absorbance wavelength of iron saturated p97</u>

p97 and transferrin bound with saturating amounts of iron have similar characteristic absorbance peaks between 460-475nm (Aisen, 1994). This absorbance profile can be used to study the iron-binding characteristics of p97 and to establish whether soluble p97 binds to one or two atoms of iron by observing the change in the absorbance at a specific wavelength as the protein is gradually saturated with iron.

Previously, Baker *et al.* reported the peak absorbance wavelength of naturally occurring soluble p97 to be 464 nm (Baker et al., 1992). However, the soluble p97 used in this thesis was a recombinant p97 made by genetically deleting the GPI-anchored signal sequence. Therefore, the peak absorbance wavelength of the recombinant protein had to be ascertained by saturating soluble p97 with excess amounts of iron nitrilotriacetate (Fe-NTA). The iron-saturated protein, which has pinkish-orange color, was subjected to a UV-visible wavelength scan from 380 nm to 600 nm. A peak absorbance was observed at 475 nm when soluble p97 was saturated with iron whereas apo-p97 did not exhibit any peak absorbance during the scan (Fig 8).

C. <u>Results II: Iron-binding titration for soluble p97</u>

After the peak absorbance of iron saturated soluble p97 was determined, the aposoluble p97 was titrated with increasing amounts of Fe-NTA. The iron titration curve indicates that the increase in absorbance at 475 nm ceases at a ratio close to 2 moles of Fe-NTA per mole of soluble p97 (Fig. 9B). This is similar to iron titration profile of human serum transferrin for which the increase in absorbance also ceases at the same



Figure 8: UV-Visible absorption spectroscopy of soluble human p97. Holo p97 absorbs at 475 nm whereas apo-p97does not have an absorbance peak at 475 nm. This is a representation of three separate experiments.



Figure 9: Iron nitrilotriacetate (Fe-NTA) titration of human serum transferrin and p97. (A) 46 moles of apo-human serum transferrin and (B) 20 moles of apo-human soluble p97 was gradually titrated with Fe-NTA. The absorbance of iron-protein complex was measured at 475 nm for human serum transferrin and at 465 nm for human soluble p97.

ratio (Fig. 9A). Therefore, the UV-visible spectrum shows that under iron saturating conditions, soluble p97 could bind to two atoms of iron.

D. <u>Discussion</u>

The four amino acid substitutions in the C-lobe of human p97 raise the question as to whether the C-lobe of the protein is able to bind iron. Therefore, UV-Visible spectroscopy study of iron binding to p97 was carried out. The absorption spectroscopy study reveals that soluble p97 has a peak absorption wavelength at 475 nm (Figure 8). This transition is due to the charge transfer from tyrosyl oxygen of the two tyrosines to bound ferric iron (Aisen, 1994) and gives holo-soluble p97 a characteristic orange-pink color in solution rather than the colorless appearance of apo-soluble p97. The λ_{max} previously reported for naturally secreted soluble p97 is 465nm, which is a 10 nm shift in λ_{max} compared to 475 nm (Baker et al., 1992). A possible explanation for the difference between the two λ_{max} is that the soluble p97 used in Baker *et al.* is a naturally secreted protein from SK-MEL 28 cells, whereas the soluble p97 used in this study is a recombinant p97, produced by eliminating the GPI-anchored signal, and expressed the protein in baby hamster kidney cells (Yang, 2000).

Iron nitrilotriacetate (FeNTA) titration of soluble p97 at 475 nm shows that two atoms of can be bound to soluble p97 (Figure 9). Thus, the interpretation that the minor lower band in lane 6 of Figure 6 can be p97 loaded with 2 atoms of iron would agree with the data from Figure 8. It is possible that the iron binds to the C-lobe of soluble p97 with low affinity. An interesting observation from the absorption spectra analysis of the D63S human serum transferrin (a mutation which mirror the D421S in the C-lobe of p97) shows that the recombinant protein is able to bind iron and results in a peak in the absorption spectra of the proteins (He et al., 1997). A possible explanation for this observation is that serine is able to form a hydrogen bond with a water molecule, which is able to provide the coordination for iron (Faber et al., 1996; He et al., 1997). However, the absence of the aspartic acid in the recombinant serum transferrin may result in a failure to lock in the iron-pocket in the C-lobe (He et al., 1997). Therefore, the binding affinity of iron for the recombinant human serum transferrin is expected to be weak. Similarly, titration of iron can bind to the soluble p97. This would suggest that the C-lobe of soluble p97 is able to bind iron although D421S substitution may have resulted in a weakened binding affinity.

III. Differential scanning calorimetry studies on iron binding to soluble p97

A. <u>Rationale</u>

The binding of ligands to macromolecules such as proteins, nucleic acids and polymers can lead to conformational change in the macromolecule structures. It has been shown that the equilibrium between the folded and unfolded state of the macromolecule is related to the difference in free energy of ligand binding between the two states (Schellman, 1975). Calorimetry has been used to measure the energy of macromoleculeligand interaction (Brandts and Lin, 1990). Isothermal titration calorimetry and differential scanning calorimetry are the two different calorimetric techniques used to determine the energy of the interactions between the macromolecule and the ligand. The isothermal calorimetry method is used to characterize the binding interactions of a macromolecule to a ligand at a constant temperature, whereas the differential scanning calorimetry method measures the conformational energy of a molecule over a range of temperatures.

Direct measurement of the heat of reaction is one of the best ways to characterize the thermodynamics of binding. Data obtained from isothermal titration calorimetry allows rapid determination of binding affinity. However, the largest binding constant that can be measured reliably by isothermal titration calorimetry is about 10⁹M⁻¹ (Haynie, 2001). This poses a problem if the binding constant is large under physiological conditions such as in the case of iron binding to the members of the transferrin family. An alternative method to measure large binding constant is differential scanning calorimetry. The parameters measured in the differential scanning calorimetry method allow the investigator to calculate the binding constant of a ligand to a macromolecule by taking advantage of a series of equations derived by Brandts and Lin (Brandts and Lin, 1990). Appendix II contains a simplified version of the derivation of the equation used to measure the binding constant of ligand to macromolecule.

The conventional method for measuring the binding constant of a ligand to a macromolecule is by equilibrium dialysis. Differential scanning calorimetry has two important advantages over the conventional equilibrium method. First, differential scanning calorimetry allows the determination of the binding constants of an individual domain in a multi-domain molecule. Secondly, although differential scanning calorimetry may be criticized as an indirect method of measuring the binding constant, it measures large binding constants (Brandts and Lin, 1990; Lin et al., 1994). Direct equilibrium methods often fail because they lack the sensitivity if the binding constant is

too large due to limitation imposed on concentrations of both the ligand and macromolecule. In addition, in an ultra-tight binding situation, the on rate is often faster than the off rate. Therefore, thermodynamic equilibrium between the ligand bound and unbound state may take months to achieve. Differential scanning calorimetry, on the other hand, avoids the need to lower the concentration of macromolecule and ligand as the interaction becomes stronger or the need to wait until thermodynamic equilibration is reached.

Brandts and Lin have derived a series of thermodynamic equations to determine the binding constants of macromolecule-ligand interactions using the parameters measured from differential scanning calorimetry (Brandts and Lin, 1990). The binding constant and the heat capacity of binding determined from the differential scanning calorimetry methods agree quantitatively with the corresponding estimates obtained from the classical equilibrium method extrapolated to the same temperature (Brandts and Lin, 1990). After weighing out the advantages and disadvantages, differential scanning calorimetry was used in this thesis to determine the binding constant of iron to soluble p97.

During a differential scanning calorimetry experiment, several parameters are measured as the protein is subjected to a temperature scan. Figure 10A depicts a simple diagram of the inside of a differential scanning calorimeter. The sample cell is filled with a protein solution and the reference cell is filled with an identical volume of solvent. During a differential scanning calorimetry experiment, the sample and the reference cells are heated over a range of temperature. As the protein solution undergoes the unfolding process (and dissociation process, if ligand is bound), energy is either absorbed or





Figure 10: (A) Sketch diagram of the inner chamber of a typical differential scanning calorimeter. Sample cell containing the protein solution and reference cell containing the buffer respectively. During a thermoscan, power is supplied to the heaters at a steady rate and a computer is linked to the calorimeter to monitor the temperature and regulate heat flow. (B) A typical thermogram of hen egg lysozyme. The heat capacity (Cp) is plotted against the temperature (Temp). The difference between the baseline of the folded and unfolded protein is the difference in heat

released. A thermogram is a plot of the heat capacity (C_p) of the protein solution as a function of temperature. Figure 10B is an example of a thermogram of hen egg lysozyme. The melting temperature (T_m) is the temperature at which there is equal concentration of unfolded protein and folded protein species. The area under the curve represents the total heat or enthalpy change (Δ H) for the entire process. In another words, Δ H= $\int C_p dT$, where C_p is the heat capacity. In some cases, two separate peaks are seen in the C_p versus temperature plot. The two peaks represent independent unfolding of two domains within the proteins, which can be studied separately.

For p97 to be a physiologically functional iron binding protein, it must have a high affinity for iron in order to compete for iron with serum transferrin, which binds iron at a very high binding constant. T binding affinities of iron for both lobes of the ovotransferrin and serum transferrin had been determined by differential scanning calorimetry (Lin et al., 1994). Therefore, this method was employed to study to determine the binding affinity of iron for soluble human p97.

B. Results I: Differential scanning calorimetry study of iron binding to soluble p97

Isothermal calorimetry was initially attempted to determine the binding constant of iron to soluble p97. Since the binding constant of iron to p97 is expected to be much larger that $10^{9}M^{-1}$, the effort was abandoned in favor of the differential scanning calorimetry.

The differential scanning calorimetry thermogram for apo-p97 shows a peak heat capacity at around 52°C and a trailing heat capacity plot at scanning temperature greater than 52°C suggesting that there are at least two transitions during the unfolding of p97

(Fig 11). Using the equations derived from Brandts and Lin, the heat capacity measured agrees with two possible unfolding patterns. The upper trace of the Figure 11 assumes that the apo-p97 unfolds into two domains, whereas the lower trace assumes the protein unfolds in three domains. Using the equations from Brandts and Lin, the fitted T_m and enthalpies of each domain is listed in Table V. The difference in heat capacity (ΔC_p) between the folded and unfolded apo-p97 is 6 kcal/mol/°C. The ΔC_p for each domain is estimated by taking the experimentally observed ΔC_p and dividing it by the number of domain. Therefore, in a two-domain fit, the ΔC_p for each domain is fixed at 3 kcal/mol/°C, whereas in a three-domain fit, the ΔC_p for each domain is fixed at 2 kcal/mol/°C. The total calorimetric heat of unfolding of apo-p97, obtained by integration of the experimental thermogram, is 367.5 kcal/mol. From Table V, the experimental calorimetric heat of unfolding agrees well with the sum of the Δ H obtained from the three-domain fit. Therefore, according to differential scanning calorimetry data, apo-p97 unfolds into three domains.

Figure 12 shows the differential scanning calorimetry thermograms of apo-p97 compared with several ratios of soluble p97 loaded with iron. It appeared that only one domain was shifted during the thermoscan. The T_m of this domain shift is about 30 °C, from 52.1 °C for apo-p97 to 82.4 °C for holo-p97. The shifted domain is presumed to be the conserved N-lobe of p97. Therefore, according to the differential scanning calorimetry method, only one domain of soluble p97 binds to iron, presumably to the N-lobe. There is a minimal shift in the T_m for the other two domains. Hence, these domains do not appear to bind iron.



Figure 11: Differential scanning calorimetry of the two transition unfolding states of human apo-soluble p97. (A)The upper trace depicts the assumption that the protein unfolds into two domains whereas (B) the lower trace depicts the assumption that the protein unfolds into three domains. This is a representation of three separate experiments.

Model	Tm (°C)	ΔH (kcal/mol)	ΔC _p (kcal/mol/ °C)
2-domain fit:			
peak 1	52.0	205	3
peak 2	55.9	122	3
3-domain fit:			
peak 1	52.1	188	2
peak 2	52.4	101	2
peak 3	57.3	97.5	2



Figure 12: DSC thermograms of p97 loaded with various ratio iron to p97 (Fe:p97). This is a representation of three experiments.

In summary, the differential scanning calorimetry study reveals that soluble p97 has an iron-binding constant of 2 x 10^{17} M⁻¹ at 25 °C and 2.6 x 10^{17} M⁻¹ at 37 °C, which are similar to the binding constant of iron for transferrin measured with the same method (1.1 x 10^{22} M⁻¹ (25°C) for the C-lobe and 8 x 10^{13} M⁻¹ (25°C) for the N-lobe).

Furthermore, the differential scanning calorimetry study suggests only one region of the soluble p97, the N-lobe, binds iron under the conditions used. Therefore, the issue whether p97 binds two atoms of iron remains unclear and will be discussed.

C. <u>Discussion</u>

In order for p97 to be a functional iron binding protein, it must have a high affinity for iron to be able to compete with serum transferrin, which binds iron at a very high binding constant. Differential scanning calorimetry was used to determine the binding constant of iron to p97. Apo-p97 was subjected to a thermoscan in the calorimeter to determine the number of domains that could potentially be involved in iron-binding. The thermogram of the unfolding of apo-p97 suggests that the protein unfolds in two transition states. The mathematical fitting of the total enthalpy and the T_m of unfolding associated with apo-p97 indicates that there are two possible unfolding patterns. Figure 11A shows that the fitting traces that suggest apo-p97 can unfold into three domains. Table V gives the results of the T_m and enthalpy associated with individual domains of the three domain-fit agrees better with the total experimental enthalpy measured. The reason for this could be that there are two more adjustable parameters in

the three-domain fit compared to the two-domain fit (additional T_m and enthalpy parameters associated with the third domain). The two-domain unfolding pattern suggests that N- and the C- lobes of the apo-p97 unfold into two individual domains (Figure 13A). There are two possible interpretations for the three-domain fit. Figure 13B shows that apo-p97 unfolds into the N-lobe and the open or unlocked C-lobe (due to the D421S substitution) is seen as two separate domains and Figure 13C suggests that apo-p97 can unfold into three domains, the zinc binding domain in addition to the N- and the C-lobes.

The shift in T_m when apo-p97 is loaded with iron is due to the increased stability of the protein when ligand is bound and therefore requires higher energy to unfold. The thermogram of p97 loaded with iron shows that only one domain shifts to a higher T_m during a thermoscan. The identity of the shifted domain is presumed to be the N-lobe. One way to confirm the N-lobe s identity is to perform the thermoscan with a recombinant N-lobe p97. However, since the amino acids for iron-binding in the N-lobe is conserved and the T_m of the domain shifts to a higher T_m at an iron to p97 ratio of 0.5: 1, the assumption is reasonably valid. The binding constant of iron to the N-lobe of p97 was determined to be 2.2 x $10^{17}M^{-1}$. However, since the other lobe did not shift when iron was present during the thermoscan, it is likely that the C-lobe of p97 does not bind iron efficiently. Even at 100:1 iron to p97 ratio, the apparently iron loaded C-lobe showed the same thermogram profile as the C-lobe of apo-p97 (Figure 12).

To put the binding constant of iron to human p97 in perspective, the binding constant of iron for transferrin measured by differential scanning calorimetry method is reported to be 1.1×10^{22} M⁻¹ for the C-lobe and 8×10^{13} M⁻¹ for the N-lobe (25°C). Lin

Figure 13: The three possible unfolding patterns of iron loaded soluble human p97 during a thermoscan. (A) The two lobes of p97 unfold into the N-and the C-domains. (B) The Nlobe of p97 unfolds into one domain whereas the C-lobe unfolds into two-sub-domains. (C) The N-and the C-lobes unfold into two domains and the proposed zinc-binding site unfolds into a separate domain.



et al. suggested that these binding constants were too large compared to the values obtained from the conventional equilibrium binding method because the T_m values observed for the holo-transferrin are too high (Lin et al., 1994). This is due to the fact that the heat scan rate is faster than the rate of unfolding of the protein and as a result, the unfolding process had not progressed to completion at a specific temperature before the heat scan proceeded. Similarly, the binding constant of iron to p97 could be an overestimation as well for the same reason. However, relative to the binding constant of iron for transferrin, p97 has a very high affinity for iron. Although the binding constant of iron to p97 is five log scale lower than that of iron for transferrin, in situations where transferrin is saturated such as in Hereditary hemochromatosis, p97 can bind to iron very tightly and mediates uptake of iron into cells.

Differential scanning calorimetry studies of the D63S N-terminal half molecule of human serum transferrin (the D63S substitution in human serum transferrin is the same substitution as the D421S substitution in human p97) also show that there is little if any shift between the T_m of iron free and supposedly iron-loaded form of the molecule (Lin et al., 1993). Interestingly, UV-Visible spectroscopy of the D63S N-lobe human transferrin showed a peak absorbance at 426nm, indicating that iron was bound to it (He et al., 1997). The failure of the iron bound D63S human transferrin to exhibit a T_m shift during a thermoscan could be due to a failure to lock in the C-lobe as a result of the amino acid substitution. Therefore, when subjected to a thermoscan, there is no observable change in the iron bound D63S N-lobe human transferrin. Likewise, for human p97, the presence of serine instead of aspartic acid in the C-lobe can result in the failure to lock up the domain when iron binds. The binding of iron to the C-lobe did not change the conformation of the lobe and therefore, there was no difference observed in the conformation between the iron bound and iron free C-lobe of p97. Hence, the presence of aspartic acid at position 421 is likely crucial for the tight binding of iron to the C-lobe of p97.

Chapter 4: Endocytosis of GPI-anchored p97

I. Brief overview of endocytosis

Endocytosis is an essential cellular process through which the cell regulates the uptake of extracellular nutrients, and the level of receptors or membrane proteins on the cell surface. This process is very dynamic and responds to numerous stimuli or membrane changes that occur during cell growth. During endocytosis, small invaginations of the membrane occur at specialized sites that are then pinched off into small vesicles, which are subsequently transported into the cells. The contents of the vesicles are either sorted to the degradative compartment or recycled back to the cell surface.

There are two general forms of endocytosis: phagocytosis (cellular eating), which is the uptake of large particles including microorganisms and cellular debris, and pinocytosis (cellular drinking), which refers to the ingestion of fluids and solutes. Pinocytosis can be furthered sub-divided into clathrin-dependent and clathrinindependent pathways. An example of clathrin-mediated pathway is the transferrin receptor mediated uptake of transferrin (Fig. 3). The clathrin-independent pathway includes caveolae-mediated endocytosis.

A. <u>Caveolae mediated endocytosis</u>

The name caveolae was proposed by Yamada to define the cellular morphology he observed in gall bladder endothelial cells as a small pocket, vesicle, cave or recess communicating with the outside of the cell (Yamada, 1955). Palade had earlier described similar invaginations in endothelial cells that shuttle molecules across the cell. He named them plasmalemmal vesicles (Palade, 1953). Since then caveolae have been implicated in many different cellular phenomena.

The study of caveolae has been greatly facilitated by the identification of the marker protein, caveolin (Rothberg et al., 1992). The 21-24 kDa integral membrane proteins are thought to be the principal components of caveolae and provide curvature to the shape of the invaginations. There are three distinct caveolin genes, caveolin-1, caveolin-2 and caveolin-3 (Monier et al., 1995; Rothberg et al., 1992; Scherer et al., 1996; Tang et al., 1996). While caveolin-1 and -2 are highly expressed in adipocytes, endothelial cells and fibroblasts, caveolin-3 is specifically expressed in striated muscle cells (Mastick and Saltiel, 1997; Scherer et al., 1997; Song et al., 1996). (Unless specified, caveolin in this thesis refers to caveolin-1)

Caveolin is thought to anchor the membrane through its 33 amino acids hydrophobic domain, leaving the C- and the N-termini free in the cytoplasm (Kurzchalia et al., 1994). Caveolin appears to be a cholesterol-binding protein and the integrity of cholesterol is required to maintain the oligomerization of caveolin (Li et al., 1996; Monier et al., 1995; Murata et al., 1995). Sterol binding reagent such as filipin and nystatin can be used to sequester cholesterol in membranes and thereby to modulate the endocytosis of certain ligands (e.g., cholera toxin) by the caveolae-like pathway of endocytosis (Deckert et al., 1996; Orlandi and Fishman, 1998; Rothberg et al., 1992). Caveolae have specific lipid composition and are enriched in proteins with either GPI or fatty acid modification (Zurzolo et al., 1994). These proteins and lipids appear to be concentrated in caveolae relative to the surrounding membrane. Lipids such as glycosphingolipids, sphingomyelins and cholesterol form the core of caveolae and play

an important role in recruiting modified proteins (Fielding and Fielding, 2000; Hooper, 1999; Ilangumaran and Hoessli, 1998).

The biogenesis of caveolae is a multi-steps process. The specialty lipids such as the glycosphingolipids, sphingomyelins and cholesterol that make up the lipid core of caveolae originate in the transitional region of the Golgi apparatus. GPI-anchored proteins and caveolin, which are synthesized in the endoplasmic reticulum and matured in the Golgi, are incorporated into these lipid cores and are shipped to the cell surface (Dupree et al., 1993; Lisanti et al., 1993). This assembly process of caveolae is different from the clathrin-coated vesicles. While caveolae are assembled in the Golgi apparatus and delivered to the plasma membrane, the clathrin coat is synthesized *de novo* at the sites of vesicle formation.

Maintenance of caveolae on the plasma membrane requires the constant shuttling of cholesterol and other lipid from the endoplasmic reticulum to the caveolae domain (Fielding and Fielding, 1996). Depletion of cholesterol by sterol binding reagents such as filipin and nystatin causes the disassembly of caveolin and eventually leads to the cessation of caveolae internalization (Smart et al., 1996). Recently, the study of the exocytic pathway of caveolin and caveolin-2 in MDCK cell revealed that the two caveolins can form homo- and hetero-oligomeric complexes in the endoplasmic reticulum, and that these complexes are modified during transport to the plasma membrane. Caveolin homo-oligomeric complexes are present in apical transport vesicles whereas caveolin and caveolin-2 hetero-oligomers are routed to the basolateral membrane (Scheiffele et al., 1998). This work provides the first glimpse into the possible existence of functionally specialized complexes of caveolins.

Early morphological studies suggest a role for caveolae in the transport of molecules across the endothelium (Simionescu and Simionescu, 1991). Other studies have also demonstrated that caveolae can endocytose specific ligands such as cholera toxin, bacteria and virus in various cells (Pelkmans et al., 2001; Shin et al., 2000,Orlandi, 1998 #387). The use of filipin and nystatin disrupts the structure of caveolae and inhibits the transcytosis of insulin and native albumin in endothelial cell (Schnitzer et al., 1996). These findings support the concept that caveolae plays an active role in cellular ligand transport.

The study on the internalization process of caveolae was further facilitated by GPI-anchored proteins with known ligands. One of them is GPI-anchored folate receptor, which provided the first biochemical data that caveolae could mediate the uptake of molecules (Anderson et al., 1992). GPI-anchored p97 is another model for such study because the ligand is iron. Ligands and receptors internalized by caveolae can have one of four fates as depicted by Figure 14 (for review please see (Anderson, 1998)). (1) The receptor is recycled back to the cell surface where as the ligand is transported into the cytoplasm, for example, folate and iron. (2) The receptor recycles back to the surface while the ligand is transported to the endoplasmic reticulum. Examples are cholesterol, fatty acids and viruses such as simian virus 40 (SV-40). (3) The ligand is transported across the cell while the receptor is recycled back to the plasma membrane such as the transcytosis of albumin and various plasma proteins. (4) Both receptor and ligand are sequestered in the vesicular caveolae compartment, for example alkaline phosphatase, endothelin and bradykinin and their receptors.



is transported into the cytoplasm (2) The receptor recycles back the surface while while the receptor is recycled. (4) Both receptor and ligand are sequestered in the caveolae (1) The receptor is recycled back to the cell surface where as the ligand The four different trafficking pathways of endocytosis mediated by the ligand is transported to the ER. (3) The ligand is transported across the cell vesicular caveolae compartment. Figure 14:

The formation of detached caveolae vesicles from the plasma membrane requires dynamin protein. When anti-dynamin antibody is injected into hepatocytes, numerous non-clathrin-coated flask-shaped structures resembling caveolae accumulate at the plasma membrane (Henley et al., 1998). The internalization of fluorescein-labeled cholera toxin B, which is normally mediated by caveolae, is inhibited in anti-dynamin antibody-injected cells. Electron microscopy shows that the cholera toxin remains concentrated in plasmalemmal caveolae in these inhibited cells and cannot gain access to cytoplasmic organelles (Henley et al., 1999). These data support the model that dynamin participates in the fission of caveolae from the plasma membrane.

Caveolae also play an important role in signal transduction. Caveolin itself was initially discovered as a phosphorylation target of the v-src kinase encoded by the Rous sarcoma virus (Glenney, 1989; Glenney and Zokas, 1989). A variety of signaling molecules are found concentrated in caveolae. Receptor and non-receptor tyrosine kinases such as protein kinase C, MAP kinases, PI3 kinases and others are found to be highly concentrated in caveolae as shown either by biochemical purification or by morphological localization methods (Lisanti et al., 1994; Liu et al., 1997; Liu et al., 1996; Smart et al., 1996). G proteins are also found in most caveolar preparations. There is now considerable evidence showing that caveolae are the sites of calcium storage and entry (Isshiki and Anderson, 1999; Lohn et al., 2000). Lipids such as ceramides, inositol triphosphates and inositol-phosphoglycans are produced in the caveolae in response to specific stimuli such as IL-1 β , bradykinin and insulin respectively (Liu and Anderson, 1995; Pike and Casey, 1996). With so many different signaling molecules congregated in

one location, caveolae are considered to be the place for signal integration, allowing cross talk between the different pathways and the plasma membrane.

There are confusions about the relationship between caveolae and lipid rafts. Lipid rafts are dynamic assemblies of cholesterol and sphingolipids in the exoplasmic leaflet of the lipid bilayer of the plasma membrane. The similarity in lipid composition between lipid rafts and caveolae has led to the assumption that the two entities are the same. The problem has arisen primarily due to the difficulty in isolating pure caveolae and the variation of techniques used by different investigator to purify and study lipid rafts and caveolae. The most frequently used method for isolating caveolae is based on density gradient centrifugation (Smart et al., 1995). This technique allows the separation of cellular membrane according to their density by centrifugation. Cholesterol, glycosphingolipids and sphingomyelins are widely believed to be enriched in or around caveolae (Fielding and Fielding, 2000; Hooper, 1999; Ilangumaran and Hoessli, 1998). This unique lipid composition of caveolae results in a higher lipid protein ratio and hence allows the lighter density caveolae to be separated from the other cellular membranes. However, in cells that lack caveolin such as neurons, these light density membranes are also present (Gorodinsky and Harris, 1995). Obviously, caveolin is not required for the formation of light density lipid rafts and cannot be used to defined lipid rafts. In order to resolve the confusion between caveolae and lipid rafts, the term caveolae is reserved strictly for morphologically defined cell surface invaginations which contain the marker protein caveolin (Brown and London, 1998; Simons and Toomre, 2000). The localization of a protein to caveolae must be supported by double labeled immunofluorescence and immunoelectron microscopy.

B. Endocytosis of GPI-anchored proteins

The endocytotic pathway of GPI-anchored protein has been studied with various techniques. A sizable amount of information regarding the endocytotic pathway of the GPI-anchored protein was derived from studies of the uptake of folate or folic acid by GPI-anchored folate receptor. Anderson et al. had shown that in MA-104 monkey cells, GPI-anchored folate receptor entered the cell through a process termed potocytosis (Anderson et al., 1992). Folate receptors are clustered in caveolae, where the acidification of the caveolar content causes the dissociation of folate from its receptor and folate is translocated into the cytoplasm by a putative carrier (Anderson, 1994; Anderson et al., 1992; Rothberg et al., 1992; Rothberg et al., 1990). During this process, the caveolae is thought to remain attached to the plasma membrane. However, there is evidence showing that caveolae are not stationary flask-shaped invaginations underneath the plasma membrane. They are shown to participate in intracellular trafficking (Schnitzer et al., 1996). There are examples of GPI-anchored proteins, which are internalized into the cells through clathrin-coated vesicles. Rijnboutt et al. proposed that GPI-anchored folate receptors are internalized into the cells via the clathrin-coated vesicles and reach the endosomal compartments. In addition to GPI-anchored folate receptor, other GPIanchored proteins, such as prion protein and placental alkaline phosphatase, have been shown to cluster in clathrin-coated vesicles (Harmey et al., 1995; Makiya et al., 1992; Shyng et al., 1994). Therefore, the route of endocytosis of GPI-anchored protein remains to be established. There are debates on whether GPI-anchored proteins naturally reside in caveolae or that the clustering of GPI-anchored molecules in caveolae is induced by specific antibodies (Mayor et al., 1994). Data from electron microscopy studies clearly show that GPI-anchored are clustered on the cell surface and within the caveolae (Ide and Saito, 1980). However, immunohistochemistry, by contrast, shows that some GPIanchored protein are either diffusely distributed and are clustered into caveolae depending on either the use of primary antibodies, secondary antibodies or fixatives (Cerneus et al., 1993; Jemmerson et al., 1985; Jemmerson and Low, 1987; Mayor et al., 1994; Parton et al., 1994). However, majority of the morphological and functional data support the conclusion that GPI-anchored proteins tend to cluster and associate with caveolae.

II. Confocal Immunofluorescence microscopy studies on the internalization pathway of GPI-anchored p97

A. <u>Rationale</u>

The study of the endocytosis pathway of GPI anchored p97 presents a unique opportunity to determine the route of endocytosis of GPI anchored protein and to follow the fate of its ligand, iron, in the process. GPI-anchored p97 has been shown previously to be able to bind and internalize iron (Kennard et al., 1995). The internalization of GPI-anchored p97 is both energy and temperature dependent. Since there are still debates on whether GPI-anchored proteins are internalized through caveolae or clathrin-coated vesicles, the internalization route of GPI-anchored p97 was examined.

Three independent methods were carried out to examine the endocytic pathway of GPI-anchored p97. The microscopy methods would provide a visual guide to the route of internalization. The sub-cellular fractionation method would circumvent the argument

that clustering of GPI-anchored proteins into caveolae is due to antibody cross-linking. Finally, sterol-binding reagents were used to examine whether iron uptake by GPIanchored p97 is inhibited if the caveolae structure is disrupted (Chen and Norkin, 1999; Fielding and Fielding, 2000; Orlandi and Fishman, 1998; Rothberg et al., 1992).

The results of this study reveal a new iron uptake pathway separate from those described previously.

B. <u>Results I: GPI-anchored p97 is internalized through caveolae</u>

In order to determine whether p97 co-localizes with caveolae or clathrin coated pits, a double-labeled immunofluorescence microscopy study of SK-MEL28 cells was performed. SK-MEL-28 is a human melanoma cell line, which expresses transferrin receptor, caveolin, clathrin and elevated levels of p97.

Cells were grown on coverslips to 70% confluency and stained with monoclonal antibodies against human p97 and transferrin receptor. Excess antibodies were removed before transferring the cells into the 37 °C chamber to allow for endocytosis for different periods of time. The cells were then fixed, permeabilized and visualized with secondary antibodies conjugated to either Alexa-488 or Alexa-546 fluorophores. Figure 15A shows that GPI-anchored p97 did not endocytose through clathrin vesicles during the endocytic process. Instead, a large amount of GPI-anchored p97 co-localized with caveolin during the endocytic process (Fig. 15B). The majority of p97 was localized to the membrane patches that contained caveolin at the 0-minute time point. At 10 minutes, a small number of p97 molecules were internalized into caveolae and subsequently at 20 minutes, most of the p97 was observed inside caveolae. As a control, the co-localization of

Figure 15: Confocal immunofluorescence micrographs of the localization of GPIanchored p97 with clathrin or caveolin on SK-MEL 28 cells. SK-MEL 28 cells were labeled with antibodies against GPI-anchored p97 at 4°C. Excess antibodies were removed before internalization was allowed at 37 °C for various periods. The cells were fixed and permeabilized before incubating with antibodies against either (A) clathrincoated vesicles or (B) caveolae. Labeled GPI-anchored p97 were visualized with Alexa 488-conjugated goat anti-mouse antibodies (green) and labeled caveolae or clathrin vesicles were visualized with Alexa 568-conjugated goat anti-rabbit antibodies (red). Merged images are shown on the right. Co-localization of Alexa 488 and Alexa 568 is indicated by the yellow color in the merged panels. (C) The enlarged image of the 20 minutes merged panel of the co-localization of GPI-anchored p97 and caveolae. The scale bar represents 1µm. These confocal micrographs are representatives of three separate experiments.





(C)



Figure 16: Confocal immunofluorescence micrographs of the localization of transferrin receptor (TfR) with clathrin or caveolin on SK-MEL 28 cells. SK-MEL 28 cells were labeled with antibodies against TfR at 4°C. Excess antibodies were removed before internalization was allowed at 37 °C for various periods. The cells were fixed and permeabilized before incubating with antibodies against either (A) clathrin-coated vesicles or (B) caveolae. Labeled TfRs were visualized with Alexa 488-conjugated goat anti-mouse antibodies (green) and labeled caveolae or clathrin vesicles were visualized with Alexa 568-conjugated goat anti-rabbit antibodies (red). Merged images are shown on the right. Co-localization of Alexa 488 and Alexa 568 is indicated by the yellow color in the merged panels. The scale bar represents 1µm. These confocal micrographs are representatives of three separate experiments.




transferrin receptor with either clathrin-coated pits or caveolae was examined. Unlike p97, there was a significant co-localization of transferrin receptor to clathrin-coated vesicles during the 10- and 20-minute time points of internalization. However, after 30 minutes, most of the transferrin receptors did not co-localize with clathrin-coated vesicles (Fig. 16A). It is possible that at 30 minutes after endocytosis, the transferrin receptor has either recycled to the cell surface or has moved to different compartments such as endosomes. In any case, most of the transferrin receptors did not co-localize with caveolin at any time point following endocytosis, demonstrating that transferrin receptor enters the cell by clathrin-mediated endocytosis (Fig. 16B).

In summary, the immunostaining and co-localization data show that GPIanchored p97 is preferentially internalized through caveolae.

C. <u>Results II: Cryo immunoelectron microscopy study on the localization of GPI-</u> anchored p97

The immunofluorescence data suggest caveolae and p97 are in similar regions of the cell. However, the data does not unambiguously show that caveolae and p97 are absolutely co-localized since the size of caveolae are smaller than the spatial resolution of a confocal microscope. Therefore, cryo immunoelectron microscopy data was obtained to support the confocal immunofluorescence study described in the previous section.

Due to the sensitivity of the caveolin epitope to paraformaldehyde and glutaldehyde, various conventional electron microscopy fixation methods could not be used. Therefore, cryo-electron microscopy was employed. SK-MEL 28 cells was infused with 25% polypyrollidone in 2.3M sucrose over-night before being mounted on

the aluminum sample pin and cryo-fixed in liquid nitrogen. Thin sections were cut at -110 °C and double stained for caveolin and p97. Caveolae were detected with anti caveolin antibodies and protein A conjugated to 5 nm colloidal gold particle and p97 was detected with anti p97 antibodies and a goat-anti-mouse secondary antibody conjugated to 10 nm colloidal gold particles. The staining of secondary antibodies conjugated to gold particles alone to the cryo-sections showed that the blocking agent used, 0.5% fish skin gelatin, was adequate in preventing non specific binding of secondary antibodies to the sections. The electron micrograph in Figure 17A shows that p97 is found in vesicular structures, which also contain caveolin (see arrows). As a control, SK-MEL 28 cryo sections were also stained for transferrin receptor and β -adaptin, a component of the clathrin coat. Figure 17B shows that some transferrin receptor co-localizes with β -adaptin (see arrows). The structural preservation of the cells subjected to this technique is poor. In Figure 17A, the structural remnant resembles a mitochondrion is observed (see letter M). However, the membrane structures of the vesicular components of the cells are poorly preserved. Therefore, alternative methods were employed to further support the observations from the confocal microscopy.

D. <u>Results III: Iron uptake by GPI-anchored p97 is disrupted by sterol sequestering</u> reagents

In order to determine whether sterol-binding reagent can disrupt iron uptake by GPI-anchored p97 mediated by caveolae, both human p97 and human transferrin receptor transfected TRVb cell lines were pre-incubated with either filipin or nystatin for 30



(B)



Figure 17: Cryo immunoelectron micrograph of SK-MEL 28 cells. (A) GPI-anchored p97 labeled with 5 nm gold particles co-localizes with caveolin labeled with 10nm gold particles (see arrows). (B) Transferrin receptor labeled with 5 nm gold co-localizes with β -adaptin labeled with 10nm gold, a subunit of the clathrin coat (see arrows). Mitochondrion is labeled with M. These micrographs are representative of two separate experiments.

minutes before ⁵⁵Fe-citrate or ⁵⁵Fe-transferrin was added to the cells respectively. Figure 18 shows the effect of the sterol binding reagents on iron uptake by transferrin/ transferrin receptor and GPI-anchored p97 pathways. Both nystatin and filipin significantly decreased the internalization of iron uptake mediated by GPI-anchored p97. Filipin reduces the uptake of ⁵⁵Fe-citrate by 60% at 25 μ g/ml while nystatin reduces the uptake of ⁵⁵Fe-citrate by 70% at 250 μ g/ml (Fig. 18). Interestingly, filipin, was able to reduce the uptake of ⁵⁵Fe-transferrin by 20% whereas nystatin has little or no effect in the uptake of ⁵⁵Fe-transferrin. Therefore, the presence of cholesterol perturbing agents clearly affects the iron uptake by GPI-anchored p97. This demonstrates that GPI-anchored p97 uptake is mediated by cholesterol/ glycolipid-enriched microdomains, which include caveolae.

E. <u>Results IV: GPI-anchored p97 fractionates with caveolae by sub-cellular</u> fractionation

GPI-anchored proteins, cholesterol, glycosphingolipids and sphingomyelins can form a detergent-insoluble membrane domain in light buoyant density following a subcellular fractionation employing a sucrose step gradient (Brown and Rose, 1992; Smart et al., 1996). Therefore, in order to provide independent biochemical evidence that p97 cofractionate with caveolae and to exclude the possibility that the clustering of GPIanchored proteins in caveolae is due to receptor cross-linking by antibodies, SK-MEL-28 cells were subjected to biochemical fractionation.

Cellular lysates separated on a sucrose gradients yielded a white flocculent band, corresponding to the low density lipid complex, between the third and the fourth fractions

Figure 18: The effect of nystatin on the iron uptake by GPI-anchored p97 and transferrin receptor. P97⁺TRVb and TfR⁺TRVb cells were treated with either nystatin, filipin or the solvent DMSO before iron uptake experiment. The presence of nystatin and filipin reduces the uptake of iron by GPI-anchored p97. In comparison, filipin and nystatin have little or no effect on iron uptake through the transferrin receptor respectively. The error bars represent the standard deviations of three separate experiments.



of the gradient as previously reported (Brown and Rose, 1992; Smart et al., 1996). Protein components in the fractions were identified by Western blot analysis. Figure 19 shows that caveolin and p97 are found in fraction 3, whereas β -adaptin, which is a protein subunit of the clathrin complex, is found in fraction 8. Transferrin receptor, on the other hand, is found in fractions 7 and 8. The presence of p97 and transferrin receptor in completely unrelated and separate fractions is in agreement with the confocal immunofluorescence data and demonstrates that p97 is found in vesicles containing caveolin whereas transferrin receptor fractionates with clathrin.

The result clearly shows that GPI-anchored p97 and transferrin receptor fractionates into different density fraction. GPI-anchored p97 fractionates with caveolin, whereas transferrin receptor is detected in the same fraction as β -adaptin.

F. Discussion

A second aspect of this thesis is to establish how GPI-anchored p97 is internalized and to determine whether iron carried by GPI-anchored p97 is donated to the cell. Studies on the GPI-anchored folate receptor provided the first biochemical clue that caveolae could mediate uptake of molecules in a variety of cells. In MA106 cells, folate receptor internalizes bound folate through caveolae (Rothberg et al., 1990). However, there are controversies on whether the endocytosis of all GPI-anchored proteins is mediated by caveolae. Some reports have suggested that the clustering of GPI-anchored proteins in caveolae depends on the use of antibodies and fixatives. Others have also suggested that some GPI-anchored proteins such as prion protein and placental alkaline phosphatase enter the cells through clathrin-coated vesicles (Makiya et al., 1992; Shyng et al., 1994).



Figure 19: Western blots of subcellular fractionation of SK-MEL 28 cell lysate in a 0-40% sucrose step gradient. Eight fractions were collected from the sucrose gradient and each fraction was subjected to SDS-PAGE and transferred onto Immobilon membrane. Each membrane was probed separately for adaptin (a component of the clathrin coat complex), transferrin receptor, caveolin and p97.

Therefore, several independent experimental methods were carried out in this study to determine the endocytic pathway of GPI-anchored p97.

The immunofluorescence experiments show that in SK-MEL 28 cells, GPIanchored p97 co-localize with caveolae (Fig. 15A). This result was also supported by cryo-immunoelectron microscopy (Figure 17) and sub-cellular fractionation of SK-MEL 28 cells on sucrose gradients (Fig. 19). In addition, the iron uptake by GPI-anchored p97 was also sensitive to the presence of two sterol binding reagents, filipin and nystatin, indicating that caveolae was involved in the process.

Electron microscopy was attempted initially in order to provide definitive highresolution electron micrograph to corroborate the co-localization of GPI-anchored p97 with caveolae from the confocal immunofluorescence experiments. However, due to the sensitivity of caveolin to conventional fixatives, cryo-immunoelectron microscopy methods were used to obtain thin sections for immuno-staining. SK-MEL 28 cells were infused with cryo-preservatives, polypyrollidone and sucrose, before freezing in liquid nitrogen. This method of preservation is barely adequate to preserve cellular structures. In addition, due to the sensitivity of the caveolin to aldehydes, the cells were fixed with only 2% (w/v) paraformaldehyde. As a result, the cells were poorly fixed and the electron micrograph illustrated in Figure 17 does not show intact cellular structures such as Golgi apparatus and nucleus. Therefore, the technique was not pursued any further.

Exposure of the p97 transfected CHO cells to sterol-binding agents such as filipin and nystatin, which are known to disrupt caveolae structure, reduces the uptake of ⁵⁵Fecitrate by GPI-anchored p97 into the cells (Fig. 18). The experiments also showed that ⁵⁵Fe-transferrin uptake was reduced in the presence of filipin but not nystatin. There are

reports suggesting that cholesterol is required for clathrin-mediated endocytosis. Subtil *et al.* and Rodal *et al.* showed that the exposure of cells to an acute cholesterol-depleting drug such as β -methyl-cyclodextrin perturbed the formation of clathrin-coated vesicles (Rodal et al., 1999; Subtil et al., 1999). However, others have shown that the use of filipin and nystatin does not affect the formation of clathrin-coated pits in the cells (Deckert et al., 1996). However, Figure 18 shows that filipin reduced the uptake of ⁵⁵Fe-transferrin by transferrin receptor and nystatin has little effects on ⁵⁵Fe-transferrin uptake by transferrin receptor. One possible explanation for this apparent dichotomy of the effect of cholesterol on clathrin-mediated pathway is that the sequestration of cholesterol by β -methyl-cyclodextrin (Subtil et al., 1999). The different effect of nystatin and filipin on ⁵⁵Fe-transferrin receptor is more likely due to the difference in the mechanism of actions of the two sterol binding reagents.

III. GPI-anchored p97 traffics to endosomes after internalization.

A. <u>Rationale</u>

There is evidence that some GPI-anchored proteins such as GPI-anchored diphtheria toxin receptors and GPI-anchored CD 14 are localized to endosomes after they are internalized (Poussin et al., 1998; Skretting et al., 1999). However, there are also contrary data suggesting that GPI-anchored proteins are not found in the endosomes (Rothberg et al., 1990). Therefore, the study of the endocytic pathway of GPI-anchored p97 is needed to determine whether GPI-anchored p97 is trafficked to the endosomes.

The localization of p97 to the endosomes will provide additional support for the model that proteins inside caveolae can be trafficked into the endocytic pathway of the cell.

B. <u>Result I: GPI-anchored p97 is trafficked to the endosomes</u>

In order to determine whether vesicles carrying GPI-anchored p97 mature into endosomes, SK-MEL 28 cells were co-stained for p97 and the early endosome marker EEA1. Similar to the previous experiment in Figure 15, SK-MEL 28 cells were incubated with antibodies against p97 for 30 minutes at 4°C. Excess anti-p97 antibodies were removed before the cells were allowed to undergo endocytosis at 37°C for various periods of time. The cells were fixed, permeabilized and incubated with anti-EEA1 antibodies. Since it is known that transferrin receptor can be found in early endosomes after it is internalized, transferrin receptor was again used as a positive control for the experiment. In the merged panels of Figure 20A, a significant number of transferrin receptors is shown to localize to endosomes 10 minutes after internalization and remains localized to the endosomes up to 30 minute after internalization. This is demonstrated by the yellow color when both EEA-1 and p97 co-localized. A small number of GPIanchored p97 co-localizes with endosomes approximately 10 minutes after p97 is internalized. At 20 minutes after internalization, a large proportion of p97 co-localizes with EEA1 containing vesicles. This co-localization pattern of p97 with EEA1 persists to 30 minutes after internalization (Fig. 20B).

Therefore, the result clearly shows that GPI-anchored p97 is trafficked to the endosomes after internalization.

Figure 20: Confocal immunofluorescence micrographs of the localization of the endosome antigen 1 (EEA1) transferrin receptor (TfR) and GPI-anchored p97 in SK-MEL 28 cells. SK-MEL 28 cells were labeled with antibodies against either (A) TfR or (B) GPI-anchored p97 at 4°C. Excess antibodies were removed before internalization was allowed at 37°C for various periods. The cells were fixed and permeabilized before incubating with antibodies against EEA1 Labeled GPI-anchored p97 and TfR were visualized with Alexa 488-conjugated rabbit anti-mouse antibodies (green) and labeled EEA1 were visualized with Alexa 568-conjugated donkey anti-goat antibodies (red). Merged images are shown on the right. Co-localization of Alexa 488 and Alexa 568 is indicated by the yellow color in the merged panels. (C) The enlarged image of the merged panel of the 20 min co-localization of GPI-anchored p97 and EEA1. The scale bar represents 1µm. These confocal micrographs are representatives of three separate experiments.



EEA1

TfR

Merged



p97

Merged

(C)



C. <u>Result II: GPI-anchored p97 traffics to the endosomes more slowly than</u> transferrin receptor

The results of the co-localization of p97 and EEA1 (Fig. 20) suggest that the majority of GPI-anchored p97 traffics to endosomes 20 minutes after internalization, whereas, transferrin receptor traffics to endosomes 10 minutes after internalization. In order to determine whether GPI-anchored p97 and transferrin localized to early endosomes at the same time, a triple labeling experiment was performed. The experiment allowed the location of EEA-1 antigen, the internalization of transferrin and GPI-anchored p97 to be followed. Serum transferrin was removed from SK-MEL 28 cells before they were incubated with Alexa 488 conjugated transferrin and antibodies against GPI-anchored p97 at 4°C. Excess antibodies and Alexa 488 conjugated transferrin were removed and the cells were allowed to undergo endocytosis at 37°C before they are fixed and permeabilized. Anti-EEA1 antibodies were used to localize the early endosomes in the cells. At 0 minute, both p97 and transferrin receptor were present at the cell surface of SK-MEL 28 whereas EEA1 exhibited a typical punctate endosomal staining (Fig. 21). At 10 minutes, most of the transferrin receptor was endocytosed into the cells and colocalized with endosomes, whereas most of p97 was still present on the cell surface. A small number of GPI-anchored p97 molecules were seen in the endosomes containing transferrin receptor. The co-localization of three fluorescence dyes (Alexa 488, Alexa 568 and Cy5) was observed as a white punctate staining in the merged image (arrows). At 20 minutes after endocytosis, the majority of transferrin receptors was no longer present in the endosomes, however, there is an increase in the number of p97 molecules associated with endosomes as shown by the pink color (when Alexa 568 co-localized Figure 21: Confocal immunofluorescence micrographs of the localization of the endosome antigen 1 (EEA1) transferrin receptor (TfR) and GPI-anchored p97 in SK-MEL 28 cells. (A) SK-MEL 28 cells were labeled with Alexa 488 conjugated human transferrin (Tf) and antibodies against GPI-anchored p97 at 4°C. Excess Alexa 488 conjugated Tf and antibodies were removed before internalization was allowed at 37°C for various periods. The cells were fixed and permeabilized before incubating with antibodies against EEA1 Labeled GPI-anchored p97 were visualized with Cy5conjugated rabbit anti-mouse antibodies (blue) and labeled EEA1 were visualized with Alexa 568-conjugated donkey anti-goat antibodies (red). Alexa 488 conjugated Tf was visualized directly as green color. Merged images are shown on the right. Co-localization of Cy5- and Alexa 568-conjugated antibodies is indicated by the pink color in the merged panels and co-localization of Cy5-conjugated antibodies and Alexa 488-conjugated Tf is visualized as green color in the merged panels. The co-localization of Alexa 488, Alexa 568-conjugated antibodies and Cy5-conjugated antibodies is visualized as white color in the merged panel (see arrows). (B) The enlarged image of a cell from the 10-minutes time point merged panels. The arrows point to the co-localization of all three fluorochromes. (C) The enlarged image of a cell from the 30-minutes time point merged panel. Colocalization of EEA1 and p97 is indicated by he pink punctate staining. The scale bar represents 1 µm. These confocal micrographs are representatives of three separate experiments.

0 min



10 min



20 min



30 min



Alexa 488 conjugated Tf

EEA1

p97

Merged



(C)



(B)

with Cy5). At 30 minutes, there were a large number of endosomal vesicles containing p97. Therefore, GPI-anchored p97 was internalized more slowly than transferrin receptor.

D. <u>Discussion</u>

GPI-anchored p97 represents the second only example where the delivery of a ligand by GPI-anchored protein is followed during the endocytic process. The internalization of GPI-anchored p97 into caveolae occurred between 10-20 minutes after the initiation of the 37°C endocytosis period. At 30 minutes after internalization, p97 co-localized with endosomes, which were labeled with EEA1 (Fig. 20). Therefore, the confocal micrograph revealed that after p97 is internalized via caveolae and trafficked to the early endosomes, thus implying the intersection of caveolae and endocytosis pathway. Specifically, most of the transferrin is shown to move into endosomes after 10 minutes at 37°C, whereas the bulk of GPI-anchored p97 traffics to the endosomes after 20 minutes. At 30 minutes, while most of the transferrin is not seen in the endosomes any longer, the majority of GPI-anchored p97 is still localized into the endosomes (Fig. 21). This would suggest that the trafficking of transferrin/ transferrin receptor complex is faster than GPI-anchored p97.

In contrast, there is evidence to support the notion that endosomes do not play a role in the trafficking of some GPI-anchored proteins into the intracellular compartments of the cells (Nichols et al., 2001). Early endosome function requires the GTPase rab5, and expression of the GDP-bound form of rab5 reduces the uptake of transferrin by the transferrin receptor. Nichols *et al.* expressed the GDP-bound form of rab5 and showed

that the absence of rab5 activity did not impair the trafficking of GPI-anchored cholera toxin from the plasma membrane to the Golgi (Nichols et al., 2001). It is also possible that iron uptake by GPI-anchored p97 is not affected by the GDP-bound form of rab5. This can be tested by transfecting the GDP-bound form of rab5 construct into SK-MEL 28 cells and determine whether rab5 is required for iron uptake by GPI-anchored p97.

Recently, a new compartment along the caveolae internalization pathway has been described in Simian virus-40 (SV-40) infected cells. The SV-40 virus was shown to infect the cells through caveolae and was trafficked to the endoplasmic reticulum. The virus entered a newly identified intermediate organelle (termed the caveosome) instead of early endosomes before proceeding to the endoplasmic reticulum (Pelkmans et al., 2001). Caveosome is defined as a pre-existing , caveolin rich intracellular organelles with a pH of ~7.0; it seems to be unique to the caveolar uptake pathway . This raises the question as to whether GPI-anchored protein is targeted to this new compartment along the endocytic pathway. However, since the internalization pathways mediated by caveolae have several fates (Fig. 14). It is possible that the circumvention of the endosomal compartments only applies to a subset of caveolae-mediated pathways. Viral entry and protein internalization are separate events and may have different targeting pathways inside the cells.

IV. The fate of iron transported by GPI-anchored p97

A. <u>Rationale</u>

The study of iron transported by GPI-anchored p97 represents a unique opportunity to follow the fate of the ligand transported by a GPI-anchored protein. As mentioned previously, the best-characterized ligand of a GPI-anchored protein is folate.

However, even in the GPI-anchored folate receptor system, it is not known how the folate escapes the caveolae, and whether endosomal compartments are involved in the uptake process.

Although it has been shown that GPI-anchored p97 can internalize iron in a time and energy dependent manner (Kennard et al., 1995), others have suggested that p97 does not donate its iron to ferritin (Richardson and Baker, 1991a; Richardson, 2000; Richardson and Baker, 1991b). In the next set of experiments, the fate iron internalized by GPI-anchored p97 will be examined. The sub-cellular fractionation data will show that the ligand, iron, is indeed found in the caveolae fraction in ⁵⁵Fe-NTA labeled cells and using immunofluorescence GPI-anchored p97 is shown to traffic to the Nramp2 containing vesicles. Immunoprecipitation of ferritin from ⁵⁵Fe-NTA labeled cells will demonstrate that iron bound to p97 is subsequently donated to ferritin.

B. <u>Results I: Iron internalized by GPI-anchored p97 is fractionated into caveolae</u> fraction

In order to determine whether iron can be found in the caveolae fraction, SK-MEL 28 cells were labeled with either ⁵⁵Fe-nitrilotriacetic acid (⁵⁵Fe-NTA) or ⁵⁵Fe-transferrin for 15 minutes. The cells were then dounce homogenized and subjected to sub-cellular fractionation employing a sucrose density gradient as previously described in Figure 19. Each fraction was immunoprecipitated with antibodies against p97 or transferrin receptor and the radioactivity associated with respective proteins was determined. The amount of iron associates with GPI-anchored p97 in each fraction is shown in Figure 22. As illustrated in the previous sub-cellular fractionation experiment

Figure 22: Iron transported by GPI-anchored p97 fractionates into caveolae fraction. SK-MEL 28 cells were incubated with either (A) ⁵⁵Fe nitrilotriacetate (⁵⁵Fe-NTA) or (B) ⁵⁵Fe-NTA loaded human transferrin. The cell lysates were subjected to sub-cellular fractionation on sucrose gradient. Each fraction was immunoprecipitated for either p97 in (A) or transferrin receptor (TfR) in (B). As a control, normal rabbit serum (NRS) is used to immunoprecipitated each fraction. The radioactivity associated with each fraction is plotted. These graphs are representatives of two separate experiments.





(Fig. 19), caveolae is detected in fraction 3 of the sucrose density gradient. The majority of the ⁵⁵Fe-NTA associated with GPI-anchored p97 is also found in fraction 3 (Fig.22A). Therefore, caveolae appear to be involved in the transportation of iron internalized by GPI-anchored p97. As a control, ⁵⁵Fe-transferrin labeled SK-MEL 28 cell lysates were subjected to sub-cellular fractionation and the results show that ⁵⁵Fe-transferrin does not appear in any specific compartment (Fig. 22B).

C. <u>Results II: GPI-anchored p97 can be localized to vesicles containing Nramp2</u>

Nramp2 is the metal ion transporter found in late endosomes or early lysosomes where iron is thought to be transported across the membrane into the cytoplasm and be donated to ferritin (Tabuchi et al., 2000). It is also a well-described marker for the late endosomes and the lysosomes (Griffiths et al., 2000; Tabuchi et al., 2000). Using immunofluorescence microscopy, I have observed that GPI-anchored p97 is trafficked to the early endosomes (Figs. 20 and 21). It is possible that GPI-anchored p97 is subsequently trafficked to the late endosomes, where Nramp2 is localized. If that is the case, then Nramp2 could mediate the egress of iron into the cytoplasm.

In order to determine the possible mechanism of how iron transported by GPIanchored p97 can be transported into the cytoplasm, SK-MEL 28 cells were transfected with a plasmid, which expresses human Nramp2 fused with enhanced green fluorescence protein (pEGFP-hNramp2). The cells expressing the fusion protein were immuno-stained with anti-p97 antibodies to detect p97. A time-course profile of GPI-anchored p97 internalization pathway co-localizing with Nramp2 is shown in Figure 23A. Transferrin receptor has previously been shown to co-localize with Nramp2 and hence was used as a Figure 23: Confocal immunofluorescence micrographs of the localization of GPIanchored p97 or transferrin receptor (TfR) with Nramp2 on SK-MEL 28 cells. SK-MEL 28 cells were transfected with Enhanced green fluorescence protein conjugated human Nramp2 (pEGFP-hNramp2) 24 hours before incubating with antibodies against either (A) TfR or (B) GPI-anchored p97 at 4°C. Excess antibodies were removed before internalization was allowed at 37°C for various periods. The cells were fixed and permeabilized before incubating with Alexa 568-conjugated goat anti-mouse antibodies (red). The EGFP-conjugated Nramp2 is visualized as green color. Merged images are shown on the right. Co-localization of EGFP-conjugated hNramp2 and Alexa 568 is indicated by the yellow color in the merged panels. (C) The enlarged image of the 20 minutes merged panel of the co-localization of GPI-anchored p97 and EGFP-hNramp2. The scale bar represents 1 μ m. These confocal micrographs are representatives of two separate experiments.









control in this experiment (Fig. 23B) (Tabuchi et al., 2000). Co-localization of GPIanchored p97 with Nramp2 occurred at 20 minutes after internalization (as shown by the yellow merged color) and continues for another 10 minutes at 37°C (arrows). The data shows that GPI-anchored p97 is in the same vesicles as Nramp2 after being taken up by the cells. Co-localization of the two proteins suggests that Nramp2 could serve as the exporter for iron taken up by GPI-anchored p97, and also that p97 likely appears in the late endosomes or lysosomes.

D. Results III: Iron internalized by GPI-anchored p97 is donated to ferritin

In order to address whether the iron internalized through GPI-anchored p97 is incorporated into ferritin, ferritin from ⁵⁵FeNTA labeled p97⁺ or p97⁻ transferrin receptor deficient TRVb cell line was immunoprecipitated in order to determine whether ⁵⁵FeNTA is associated with the protein. These cells were also treated either with or without phosphoinositol-phospholipase C (PI-PLC), an enzyme that cleaves GPI-anchored protein to ensure that the iron loading of ferritin is dependent on the intact GPI-anchored p97. Figure 24A shows that when p97⁺TRVb cells were treated with PI-PLC, the amount of ⁵⁵Fe associated with GPI-anchored p97 decreased. This corresponds to a decrease in ⁵⁵Fe incorporated into ferritin. As a control, transferrin receptor deficient TRVb cells transfected with human transferrin receptor (TfR⁺TRVb) were labeled with ⁵⁵Fetransferrin. The cells, treated with or without PI-PLC, were immunoprecipitated with antibodies against transferrin receptor and ferritin (Fig. 24B). There was no significant difference in ⁵⁵Fe incorporation of ferritin between the PI-PLC treated or untreated cells since transferrin receptor is not sensitive to PI-PLC treatment. Figure 24: GPI-anchored p97 is able to donate iron to ferritin. Endogenous transferrin receptor negative TRVb cell, and those transfected with human p97 (p97⁺TRVb) and human transferrin receptor (TfR⁺TRVb) were treated either with or without phosphoinositol-phospholipase C (PI-PLC) before they were incubated with (A) ⁵⁵Fe nitrilotriacetate (⁵⁵FeNTA) for the p97⁺TRVb cells or (B) ⁵⁵FeNTA loaded human transferrin for the TfR⁺TRVb cells. The cell lysates were pre-cleared and immunoprecipitated with antibodies against normal rabbit serum (NRS), ferritin and p97 (L235) (for the p97⁺TRVb cells) or transferrin receptor (OKT9) (for the TfR⁺TRVb cells). The radioactivity count associated with the immunoprecipitate was plotted. The error bars represent the standard deviations of three separate experiments.





Interestingly, in ⁵⁵Fe labeled p97 TRVb cells, there is a significant expression of an endogenous protein, likely hamster p97, which could bind ⁵⁵Fe and was sensitive to PI-PLC treatment. When these TRVb cells were treated with PI-PLC, the amount of ⁵⁵Fe associated with this protein decreased, which corresponded to a decrease in ⁵⁵Fe incorporation into ferritin.

In summary, the results demonstrate that iron bound to GPI-anchored p97 fractionates into caveolae fraction and is subsequently incorporated to ferritin. GPIanchored p97 is in the same vesicular compartments as Nramp2, which would suggest a possible route for iron egress into the cytoplasm.

E. <u>Discussion</u>

It has been proposed by several investigators that Nramp2 expressed on endosomal membrane is the channel for iron taken up by transferrin/ transferrin receptor to egress into the cytoplasm and ultimately to be incorporated into ferritin (Burdo et al., 1999; Fleming et al., 1998). Using confocal immunofluorescence techniques, the route of endocytosis of GPI-anchored p97 was followed through to the endosomal compartments and vesicles containing the Nramp2 protein. Therefore, it is possible that iron taken up by p97 can be released into the cytoplasm by Nramp2. This provides a new understanding of a novel route of iron uptake through caveolae and ligand internalization by GPIanchored protein. Previous report showed that Nramp2 is localized to the recycling endosomes and lysosomes (Tabuchi et al., 2000). Hence, a significant number of p97 appears to traffic from the early endosomes to the recycling endosomes or the lysosomesafter it is internalized. A model of iron internalization pathway by GPI- anchored p97 is illustrated in Figure 25. The iron bound to GPI-anchored p97 is internalized through caveolae and traffics through from early endosomes to recycling endosomes or lysosomes where Nramp2 resides. Since GPI-anchored p97 endocytose more slowly than transferrin receptor, only a smaller number of GPI-anchored p97 associates with transferrin receptor in the endosomes compartment. A majority of the GPI-anchored p97 enters the endosomes at a later stage. Iron dissociates from p97 and is transported by Nramp2 into the cytoplasm where it is incorporated into ferritin.

Recently, it was pointed out that p97 does not have an iron response element (Richardson, 2000). Therefore, its expression is likely not regulated by the level of intracellular iron, similar to the transferrin receptor 2, which does not possess an iron response element (Kawabata et al., 2000). Other researchers have also suggested that p97 does not play a substantial role in iron uptake in HeLa cells (Kriegerbeckova and Kovar, 2000). It has been shown that HeLa cells have few if any caveolae (Skretting et al., 1999). Therefore, studies of the internalization of p97 and any other GPI-anchored molecules in these cells might be impeded.

In previous studies using SK-MEL 28 cells, it was suggested that while a membrane component identified on SK-MEL 28 consistent with p97 could bind iron, it did not appear to donate iron to the cell (Richardson and Baker, 1991a; Richardson and Baker, 1991b). The experiments were performed on the SK-MEL 28 cell line without a p97 negative control cell line. The results in Figure 22A clearly indicate that irons internalized by GPI-anchored p97 can be localized to caveolae fractions and they are subsequently donated to ferritin (Figure 24). When human p97 transfected transferrin receptor deficient TRVb cells were treated with PI-PLC, the amount of iron donated by

Figure 25: The proposed model of iron uptake pathway by GPI-anchored p97. (A) GPIanchored p97 bound with Fe3+ is internalized via caveolae. As a comparison, transferrin receptor (TfR) mediated iron-transferrin uptake is shown. Holo-transferrin bound to transferrin receptor is endocytosed through clathrin-coated pits. The caveolae and the clathrin-coated vesicles either mature into or fuse with endosomes separately. Iron released from p97 in the endosomes escapes the late endosome through the divalent metal transporter Nramp2 and incorporated into ferritin in the cytoplasm. (B) GPI-anchored p97 and TfR are endocytosed into the same endosomal compartments. Iron released from transferrin/ transferrin receptor complex and GPI-anchored p97 escapes the late endosomes through the divalent metal transporter Nramp2 and incorporated into ferritin in the cytoplasm.


p97 to ferritin decreased in correspondence to the decrease in iron uptake due to the removal of GPI-anchored p97 by PI-PLC. Interestingly, p97⁻ transferrin receptor deficient CHO cell line, which does not express a functional transferrin receptor, is able to accumulate iron in ferritin. Presumably, this cell line can take up iron by either internalizing non-specifically bound ⁵⁵Fe-NTA or by a specific membrane bound channel or carrier (Egyed, 1988). The data also indicated that this specific component was sensitive to PI-PLC treatment. It has been suggested that this PI-PLC sensitive component could be the GPI-anchored hamster p97 (Kennard et al., 1995). Experiments to determine whether this component is hamster p97 are in progress.

In summary, the internalization pathway of iron by GPI-anchored p97 was followed by a series of time-course confocal immunofluorescence and sub-cellular fractionation experiments. From these experiments, it was determined that GPI-anchored p97 is internalized through caveolae in SK-MEL 28 cells and is subsequently trafficked to endosomes and vesicles containing Nramp2. Iron internalized by GPI-anchored p97 is found in caveolae containing fractions of the sucrose gradients and is incorporated into ferritin. These results establish iron taken up by GPI-anchored p97 as a new pathway for cellular iron acquisition.

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Chapter 5 Concluding remarks and future perspectives

There is renewed interest in the study of iron metabolism. The recent cloning of several proteins involved in iron metabolism including basal lateral iron transporter (IREG1), transferrin receptor 2, duodenum ferric reductase (Dcytb) and HFE, has created a major breakthrough in the understanding of the mechanisms of iron absorption, iron transport and cellular regulation of iron metabolism. Iron is an essential nutrient for cellular metabolism. Deficiency in iron results anemic symptoms, which includes pallor, fatigue and decreased work performance. Data collected from multiple countries by the World Health Organization estimated that 50% of children and women and 25% of men in developing countries, and 7% to 12% of the population in developed countries are iron deficient (Freire, 1997). However, excess amount of iron is deleterious due to iron s role in the generation of free radicals, which can lead to tissue damage.

The best-characterized pathway for iron uptake is the transferrin receptor mediated holo-transferrin internalization through the clathrin-coated pit. Holo-transferrin binds to transferrin receptor on the cell surface and is internalized within the endosomal compartments. The hemochromatosis protein, HFE, appears to play the role of modulating the affinity of transferrin receptor for transferrin during this process. The acidification of the endosomes prompts the release of iron from the transferrin/ transferrin receptor complex. Iron is transported across the membrane into the cytoplasm by Nramp2 and is subsequently utilized for cellular metabolism or is sequestered within the iron-storage protein, ferritin. Although the interaction between HFE and transferrin receptor seems to lower the receptor affinity for transferrin by approximately 10-fold (Feder et al., 1997; Lebron et al., 1998), the precise role of HFE remains obscure. The pathology of several iron metabolism disorders has provided hints on the existence of transferrin/ transferrin receptor independent mechanisms of iron uptake. The studies of hypotransferrinemic (hpx) mouse and hereditary hemochromatosis in humans, where circulating non-transferrin bound iron can reach micro-molar levels, have propelled the researchers to identify transferrin/ transferrin receptor-independent iron transport mechanisms such as iron uptake by p97 (Batey et al., 1980; Bernstein, 1987; Buys et al., 1991; Craven et al., 1987; Gutteridge et al., 1985; McNamara et al., 1999).

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The physiological functions of p97 are just coming to light at the start of this study. GPI-anchored p97 is shown to internalize iron in p97 transfected CHO cells (Kennard et al., 1995). The association of elevated levels of serum p97 with the progression of Alzheimer's disease and the pathological roles different metals play in the disease have led to the hypothesis that p97 may play a role in the pathology of the disease by transporting metals related the into the brain (Kennard et al., 1996). Before establishing p97 as a candidate for metal transport into the brain, it was crucial to determine whether these metals could bind to p97. The identification of the orthologues of p97 in different species suggested that the function of the gene is evolutionary preserved. However, the amino acid substitutions in one of the iron binding sites (in some species, both sites are affected) has cast doubts as to whether p97 does function as an iron binding protein. In order to confirm p97 as an iron binding protein, it is important to determine whether the protein bind iron at a high affinity. Furthermore, others have suggested that p97 is not a functional iron transport protein and does not donate its iron to ferritin (Richardson and Baker, 1991a; Richardson, 2000; Richardson and Baker, 1991b).

The increased controversies regarding the physiological roles of p97 has led to the studies outlined in this thesis.

Aluminum, copper and zinc were shown to be able to interfere with iron binding to GPI-anchored p97 by the competition experiments. In addition, iron-loading experiments with soluble p97 and these different metals also demonstrated that p97 could bind to metals other than iron. However, the urea polyacrylamide gel electrophoresis of soluble p97 loaded with different metals showed that p97 bound preferentially to iron. Indeed the high binding affinity of iron for p97 determined from differential scanning calorimetry demonstrated that at least one atom of iron can bind to the N-lobe of p97 very tightly at 2.2 x 10^{17} M⁻¹. Data from urea polyacrylamide gel electrophoresis revealed that the C-lobe of p97 appears to bind iron very weakly. The titration of iron to p97 monitored by UV-visible spectroscopy also illustrated that the molar ratio of iron to p97 is 2:1. The failure to detect iron binding in the C-lobe using differential scanning calorimetry could be due to the fact that the conformation of iron bound C-lobe is no different from iron-free C-lobe. The D421S amino acid substitution in the C-lobe of p97 may have resulted in an ineffective domain closure required for tight iron binding. The observation with the D63S recombinant N-lobe transferrin also suggests that the aspartic acid to serine substitution appears to result in a failure to lock in in the iron when it is bound to the protein (He et al., 1997). Therefore, alternate method is needed to determine the iron binding constant of the C-lobe of p97.

The process of crystallizing soluble p97 is underway (see Appendix I). The crystal structure of p97 will show that whether or not the C-lobe contains iron. In

addition, by crystallizing zinc-bound soluble p97, it is possible to determine whether zinc binds to the proposed zinc-binding site (Garratt and Jhoti, 1992).

The internalization of iron bound to GPI-anchored p97 represents a unique opportunity to study another example of the uptake of a ligand bound to a GPI-anchored protein. Iron bound to GPI-anchored p97 is internalized through caveolae and is subsequently trafficked through the endosomes and the Nramp2 containing vesicles before it is incorporated into ferritin in the cytoplasm. There were doubts as to whether caveolae were able to mediate internalization of any substances. Caveolae were initially thought to form flask shape invaginations underneath the plasma membrane. These invaginations were thought to not detach from the plasma membrane and therefore some investigators suggest that caveolae are not involved in uptake of extracellular substances. However, the study of folate uptake by GPI-anchored folate receptor has altered the perception of the static nature of caveolae. The recent observations that caveolae are involved in the entry of bacteria and virus into cell have also widen the possible role of caveolae in mediating endocytosis of particles from extracellular space (Pelkmans et al., 2001; Shin et al., 2000). There are now mounting evidence suggesting caveolae, in addition to being the sites of signal integration in the cellular signaling pathways, are also the sites of calcium storage and entry (Isshiki and Anderson, 1999; Lohn et al., 2000). Furthermore, SV40 viral entry and GPI-anchored mediated bacterial internalization have also been linked to caveolae (Parton and Lindsay, 1999; Pelkmans et al., 2001; Shin and Abraham, 2001a; Shin and Abraham, 2001b; Shin et al., 2000). The uptake of iron by GPI-anchored p97 through caveolae presented in this thesis represents a novel pathway of iron acquisition and iron bound to the GPI-anchored p97 can be incorporated into ferritin.

The increased understanding of the role of caveolae play in cellular processes has over turned the previous perception that caveolae are static vesicles reside underneath the plasma membrane.

The existence of several transferrin/ transferrin receptor independent iron uptake pathways in organisms, of which p97 is one of them, raises the question as to the purpose of these pathways. The efficiency of transferrin/ transferrin receptor to deliver iron into the cells also questions the necessity of these alternate routes of iron internalization. One of the purposes of having transferrin/ transferrin receptor independent iron uptake pathway may lie with the regulation of iron uptake. Transferrin/ transferrin receptor is very efficient in amassing iron in the cells. However, this may be necessary only in red blood cells where iron is required for heme assembly or in rapidly dividing cells such as cancer cells (for review please see (Lauffer, 1992). In cells that do not have high requirement for iron, transferrin/ transferrin receptor may not be the preferred route for iron uptake. For example, in brain tissue, where very few cells undergo proliferation, iron is not a major element required. The expression of p97 on brain capillary endothelium implies that it may have functional role in mediating brain iron uptake (Rothenberger et al., 1996). In addition, the amino acid substitution in human p97, which results in only one high affinity iron-binding lobe, may serve as way to limit the amount of iron internalized by certain cells. Therefore, depending on the cellular need for iron, different iron uptake pathway may be employed and p97 may be the preferred pathway for iron uptake.

The presence of transferrin/ transferrin receptor independent iron uptake pathways in specific cells suggests specialized functions of these pathways. For example, the

presence of Nramp2 on the apical plasma membrane of intestinal epithelial cells is necessary for ferrous iron uptake. The expression of GPI-anchored p97 on the fetal pig intestinal cells implies that p97 may be involved in iron uptake during fetal development (Alemany et al., 1993). The presence of these two independent iron absorption pathways can infer differential iron uptake. Dietary iron is in ferric form and has to be reduced by a ferric reductase (Dcytb) to the ferrous form before transported by Nramp2. GPIanchored p97, however, can bind and transport ferric iron. In addition, while Nramp2 seems to be a non-specific divalent metal transporter, p97 binds iron preferentially. Therefore, p97 may play a unique role in cellular iron uptake in specialized cells such as polarized cells. Future studies should include the investigations of the role of p97 in intestinal iron uptake. Furthermore, p97 has been shown to be expressed in the human epithelial cells of the sweat gland. One could hypothesize that p97 could mediate iron transport in specific tissue where the environmental salinity is different from the rest of the body.

It is clear that there are several directions this project could take in the future. For example: studies to understand whether p97 plays a role in intestinal iron uptake and iron deposition in several iron-overload disorders using mouse deficient in mouse p97 (p97 knock-out mouse) and mouse that over expresses p97. By culturing brain endothelial cell layer in a transwell apparatus, an *in vitro* model of the blood brain barrier can be set up to study the role of p97 in transcytosis metals across the monolayer. The identification of a potential receptor for soluble p97 will also improve the understanding of the physiological role of p97. Although these ideas were not discussed in details, they are important in understanding the physiological role of p97. In conclusion, the results of this

thesis show that p97 is able to bind at least one atom of iron at a very high affinity. The C-lobe of p97 is able to bind iron as well although the binding affinity is expected to be low. A novel route of iron uptake independent of transferrin/ transferrin receptor is also demonstrated. GPI-anchored p97 can serve as a functional iron transport protein, releasing iron into the cell for incorporation into ferritin. This data sheds significant new light on transferrin/transferrin receptor independent pathways and also on the fate of ligands internalized by GPI-anchored proteins.

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Appendix I: Crystallization of soluble p97

Deciphering the crystal structure of p97 will help visualizing the spatial interaction of iron with the iron-binding site of the protein. However, to solve the crystal structure of p97 is an ambitious task. The initial condition for crystal growth has been determined and optimization of the conditions is in progress. Analysis of the X-ray diffraction pattern of the protein will be carried out by a group of expertise by collaboration.

Experimental design and results: The most common setup to grow protein crystals is by the hanging drop technique. In order to pin point the exact condition required for crystal growth, two pre-made kits were used to screen for optimal condition (Hampton research Crystal screen 1 and Crystal screen 2 and Emerald Biostructures Wizard 1 screen and Wizard 2 screen). 31 of soluble p97 (17mg/ml) was mixed with 31 of reservoir solution containing the precipitants. This mixture was put on a glass cover slip and sealed over the reservoir. This method is based on the idea that as the protein/precipitant mixture in the drop is less concentrated than the reservoir solution, water evaporates from the drop into the reservoir. The concentration of both protein and precipitant in the drop slowly increases, and crystals may form.

After 4 days at 20ßC, a rectangular shape crystal was observed in the reservoir containing 20% PEG-8000, 200 mM NaCl, 100 mM CAPS pH 10.5. The crystal was orange in color, which is indicative of an iron binding protein. Measurement of the crystal showed approximate dimensions of 0.9 mm x 0.06 mm x 0.05 mm (Fig. 26).

In order to optimize the condition for crystal growth, various combinations of buffer at different pH and salt concentrations will be examined.



Figure 26: Crystal of soluble p97

Appendix II: Derivation of the equation to calculate the binding constant in terms of enthalpy, heat capacity and temperature.

The transposition of any equilibrium constant K_1 and K_2 at temperature T_1 to another temperature T_2 is derived from Gibb s free energy formula.

 $\Delta G = \Delta H(T) - T\Delta S(T)$ where H(enthalpy) and S(entropy) is a variable of temperature T

 ΔG = -RTlnK where K is the equilibrium constant and R is the gas constant

 $-RTlnK = \Delta H(T) - T\Delta S(T)$

 $\ln K = -\Delta H(T)/RT + T\Delta S(T)/RT$

 $\ln K = -\Delta H(T)/R(1/T) + \Delta S(T)/R$

Differentiate with respect to T

 $\underline{dlnK} = \Delta H(T)/R(1/T^2) + 0$

Integrate from T₂ to T₁

 $\int_{T_1}^{T_2} d\ln K = \int_{T_1}^{T_2} \Delta H(T) / R(1/T^2) dT$

T1

 $\Delta H(T) = \Delta H(T_1) + \Delta Cp(T-T_1)$ from $\Delta H = \int_T C_p dT$, where is relatively constant in a

constant pressure condition.(i.e. if C_p is relatively constant, then $\Delta H = \int_{T}^{T_1} C_p dT$ can be integrated to give an approximate expression for temperature dependence of ΔH with respect to an arbitrary reference temperature T₁)

$$\int_{T_1}^{T_2} d\ln K = \int_{T_1}^{T_2} [\Delta H(T_1) + \Delta C_p(T-T_1)] / R(1/T^2) dT$$

$$\int_{T_1}^{T_2} d\ln K = \int_{T_1}^{T_2} [\Delta H(T_1)/R](1/T^2) dT + \int_{T_1}^{T_2} \Delta C_p T/RT^2 dT - \int_{T_1}^{T_2} \Delta C_p T_1/RT^2 dT$$

$$\ln K_2/K_1 = -[\Delta H(T_1)/R] (1/T) \Big|_{T_1}^{T_2} + \Delta C_p/R(\ln T) \Big|_{T_1}^{T_2} + \Delta C_pT_1/R (1/T) \Big|_{T_1}^{T_2}$$

$$\ln K_{2}/K_{1} = -[\Delta H(T_{1})/R] (1/T_{2}-1/T_{1}) + \Delta C_{p}/R(\ln T_{2}/T_{1}) + \Delta C_{p}T_{1}/R (1/T_{2}-1/T_{1})$$

$$\ln K_{2}/K_{1} = -[\Delta H(T_{1})/R] (1/T_{2}-1/T_{1}) + \Delta C_{p}/R (\ln T_{2}/T_{1}+T_{1}/T_{2}-1)$$

$$K_{2}/K_{1} = e^{-[\Delta H(T_{1})/R] (1/T_{2}-1/T_{1}) + \Delta C_{p}/R (\ln T_{2}/T_{1}+T_{1}/T_{2}-1)}$$

$$K_{2} = K_{1}e^{-[\Delta H(T_{1})/R] (1/T_{2}-1/T_{1}) + \Delta C_{p}/R (\ln T_{2}/T_{1}+T_{1}/T_{2}-1)}$$
(1)

The following is a summary of the derivation of an equation to calculate the binding constant (K_L) of a ligand L (in this thesis L is iron) to a protein P (soluble p97). Since only one binding site is observed for iron to soluble p97 binding, the binding constant K_L is calculated using equation that fits 1:1 binding stoichiometry in a single unfolding transition (Brandts and Lin)

The following abbreviations are the same as those used in Brandts and Lin.

$P_t = Total protein$	$L_t = Total ligand$
P = Folded protein	L = Free ligand
P = Unfolded protein	[L] $_{Tm}$ = Free ligand concentration at T_m

K_L=Binding constant

K = Unfolding constant

 T_m = temperature at which there are equal concentration of folded and unfolded in the presence of ligand.

 T_o = Temperature at which there are equal concentration of folded and unfolded protein in the absence of ligand.

The reactions involved at equilibrium for the protein in the absence and presence of ligand are as followed.

$$P = P$$
 $K=[P]/[P]$ (2)
 $P + L = PL$ $K_L=[PL]/[P][L]$ (3)

At equilibrium, the concentrations of folded and unfolded protein are equal. Therefore $K_{eq}(T_o)$ and $K_{eq}(T_m)$ are equal to 1.

In the presence of ligand, at equilibrium, P = [P] + [PL]

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[P]
$$K_{eq}(T_m) = ---- = 1$$

[P] + [PL]

Substitute for [P] and [PL] using equations (2) and (3),

$$K(T_{m})[P] K(T_{m})$$

$$K_{eq}(T_{m}) = ----- = 1 (4)$$

$$[P] + K_{L}(T_{m}) [P][L]_{Tm} 1 + K_{L}(T_{m}) [L]_{Tm}$$

$$K_{L}(T_{m}) = (K(T_{m})-1)/[L]_{Tm} (5)$$

According to equation (1),

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$$K(T_m) = K(T_o) \exp \left[-\Delta H(T_o) / R(1/T_m - 1/T_o) + \Delta C_p / R(LnT_m/T_o + T_o/T_m - 1)\right] (6)$$

At equilibrium $K(T_o)=1$ (i.e. at equilibrium P=P of the protein in the absence of ligand), when the folded and unfolded proteins are in equal concentrations in the absence of ligand. Therefore,

$$K(T_{m}) = \exp \left[-\Delta H(T_{o})/R(1/T_{m}-1/T_{o}) + \Delta C_{p}/R(\ln T_{m}/T_{o} + T_{o}/T_{m}-1)\right]$$
(7)
Substitute (10) into (8)
$$K_{L}(T_{m}) = \left[\exp \left[-\Delta H(T_{o})/R(1/T_{m}-1/T_{o}) + \Delta C_{p}/R(\ln T_{m}/T_{o} + T_{o}/T_{m}-1)\right] - 1\right]/[L]_{Tm}$$
(8)

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